SYNTHETIC GENOMICS®

07 April 2019

***Electronic Delivery Via CDX***

U.S. Environmental Protection Agency
Office of Pollution Prevention & Toxics
New Chemicals Program
EPA East Building, Room 4133
1201 Constitution Avenue, NW
Washington, DC 2,0004-3302

Re: TSCA Experimental Release Application of Synthetic Genomics, Inc.
Open Pond Research and Development: The green microalgal strain *Parachlorella* STR26155 engineered with green fluorescent protein (GFP) for environmental tracking

Ladies and Gentlemen:

On behalf of Synthetic Genomics, Inc., I enclose its application for authorization to conduct the outdoor, open pond R&D activities described herein with the green microalgal strain *Parachlorella* STR26155 engineered with green fluorescent protein (GFP) for environmental tracking.

This submission represents the culmination of 18 months of investigation and data collection, in consultation with EPA, to lay the foundation for efficient review of future TERAs for future outdoor, open pond algae research and development work. Extensive and flexible outdoor testing is a critical and necessary stage of development for commercial algae cultivation intended to be conducted outdoors in the future on a large scale.

The aim of this TERA and the research for which it seeks authorization in part is to establish baseline environmental conditions in and around the test facility, and to evaluate and confirm the sufficiency of control and monitoring equipment and techniques developed for this and other similar outdoor R&D programs. To facilitate this work and appropriate public consultation, we have minimized confidential business information (CBI) claims to the greatest extent possible.

We expect that these efforts, in collaboration with EPA, will establish a more predictable framework for future EPA reviews of outdoor algae R&D activities at our test facility, presumptively sufficient to permit the Agency to make a reasoned evaluation of the health and environmental effects of the microorganism under the conditions of use. Future TERAs built on

the framework would allow EPA to streamline and focus its future reviews more narrowly on the relevant properties of the organism rather than the sufficiency of baseline information or control and monitoring techniques. With greater experience, EPA will need to develop a practicable decision framework for the research program TERAs anticipated by the regulation, balancing innovators' need for nimble flexibility during R&D with a reasonable and appropriate level of environmental protection consistent with TSCA. We hope you will review this TERA with these larger goals in mind.

We look forward to working with you.

Very truly yours,

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David Hanselman
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SYNTHETIC GENOMICS®

# *TSCA Experimental Release Application of Synthetic Genomics, Inc. - Open Pond Research and Development*

## *The green microalgal strain Parachlorella STR26155 engineered with green fluorescent protein (GFP) for environmental tracking*

**Submitter: Synthetic Genomics, Inc.**

07 April 2019

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# CERTIFICATION STATEMENT

I certify that to the best of my knowledge and belief: The company named in this submission intends to manufacture, import, or process for a commercial purpose, other than in small quantities solely for research and development, the microorganism identified in this submission. All information provided in this submission is complete and truthful as of the date of submission. I am including with this submission all test data in my possession or control and a description of all other data known to or reasonably ascertainable by me as required by 40 C.F.R. § 725.160 or § 725.260.

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David Hanselman
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_____ 07 April 2019

David S. Hanselman, Ph.D.,
Senior Director, Corporate Regulatory Affairs and Quality
Synthetic Genomics, Inc.
(Authorized Official)

# *TERA of Synthetic Genomics, Inc.*
# *Open Pond Research and Development*

## *The green microalgal strain Parachlorella STR26155 engineered with green fluorescent protein (GFP) for environmental tracking*

## Table of Contents

## Executive Summary

The purpose of this submission is to describe the establishment of a baseline program for the assessment and monitoring of potential environmental releases associated with large-scale cultivation of bioengineered algae in outdoor ponds for research and development. To this end, Synthetic Genomics, Inc. is submitting this TSCA Environmental Release Application (TERA) which describes the construction and intended cultivation and monitoring of a minimally modified *Parachlorella*. The submission:

- Describes the construction of a minimally engineered strain of *Parachlorella* and its intended deployment to open pond aquaculture for R&D at SGI's California Advanced Algae Facility (CAAF);
- Establishes that the described GFP-*Parachlorella* construct strain has no competitive advantages over the wildtype strain in respect to growth and environmental dispersal;
- Establishes that the described GFP-*Parachlorella* construct strain does not present health risks different than the wildtype strain;
- Describes a robust and extensive monitoring program utilizing the inherent and engineered characteristics of the notified GFP-*Parachlorella*;
- Provides a thorough data reporting pipeline model that will facilitate submission and review of future TERAs detailing commercially-relevant algae constructs; and
- Demonstrates that the open pond cultivation of the modified *Parachlorella* for R&D under the proposed handling and monitoring program will not present an unreasonable risk of injury to human health or the environment.

Perhaps the highest hurdle for commercially-relevant biofuel production to overcome is identifying and resolving performance and cultivation issues arising from the scale-up from controlled laboratory conditions and initial field tests under monitored ambient outdoor conditions, to full-scale open pond cultivation. The establishment of an accepted, practicable, standardized baseline assessment and monitoring program for open pond R&D will allow for more iterative changes to a production organism of interest in the R&D context, which is crucial in the development of an economically feasible path to commercial viability for algal biofuels production. Synthetic Genomics is a world recognized leader in synthetic biology, and coupled with its state-of-the-art production facility in Calipatria, CA, it is uniquely positioned to lay the groundwork necessary for the future of the industry.

# Introduction and Overview

## 1) Introduction

Synthetic Genomics, Inc.[a] (SGI) is a pioneer in the development of genomic engineering technologies and is one of the first to harness some of these next-generation genetic tools towards large-scale commercial applications. Headquartered in La Jolla, California, our approach for optimizing and customizing microbes - ones that can perform commercially useful functions - is much more efficient than incumbent technologies. The advancement of these tools and the resulting cost reductions enable industry to more cost effectively develop commercially valuable bio-products across a broad spectrum of applications.

Since 2009, SGI has collaborated with its partner ExxonMobil Corporation[b] to develop scalable, low carbon biofuels from algae. The focus of our collaboration to-date has been to identify and improve algal biocatalysts that exhibit bio-oil productivity and other characteristics needed for large scale cultivation. For the majority of this R&D effort, work has been conducted in the lab; but industrially-relevant conditions for deploying algae biofuels are very different than a lab environment. Coming on the heels of lab-scale algae biofuels R&D progress,[1-2] it is necessary to begin carefully and methodically testing the technology outdoors where it will be eventually deployed.[c]

## 2) The Benefits of Using Algae for Biofuel Production[d,e]

Algae represents a significant improvement over alternate biofuel sources for several reasons, including the following:

- Unlike first generation ethanol and biodiesel, producing algae does not compete with land used for food production as long as the land selected for cultivation is not suitable for food production, such as non-arable land;
- Algae consume carbon dioxide ($CO_2$) and use sunlight as an energy source to produce high quality oils that can replace high energy dense transportation fuels like diesel and jet fuel. These fuels will remain essential even if a large-scale deployment of electric cars and light transportation vehicles reduces the need for gasoline; and
- Algae can yield more biofuel per acre than plant-based biofuels – currently about 2,000 gallons of fuel per acre, per year.

[a] https://www.syntheticgenomics.com/

[b] https://www.businesswire.com/news/home/20180306005178/en/ExxonMobil-Synthetic-Genomics-Algae-Biofuels-Program-Targets

[c] Algae is heading to the farm. https://youtu.be/HFWdq_1LXvQ

[d] https://corporate.exxonmobil.com/en/Research-and-innovation/Advanced-biofuels/Advanced-biofuels-and-algae-research

[e] https://energyfactor.exxonmobil.com/category/science-technology/

## 3) Algae Biofuel Outdoor Program Objectives

The purpose of this TSCA Experimental Release Application (TERA) is to lay the foundations necessary to link the biology work in the lab with successful scale-up in the field by experimenting at a manageable scale. Gaining insight into how algal strains (our top candidates today as well as those we will develop as the collaboration continues) perform in industrially-relevant settings will inform the design of the technology and ultimately accelerate its development and deployment. It will also reduce the risk of failure that comes with continuing to design a technology without knowing the conditions and constraints it will ultimately face at-scale. This effort will contribute to the development of a globally-relevant Safety, Health & Environment package, or "template", for subsequent TERA and MCAN (TSCA Microbial Commercial Activity Notification) submissions to US EPA and international environmental protection agencies.

We expect that these efforts, in collaboration with EPA, will establish a more predictable framework for future EPA reviews of outdoor algae R&D activities at our test facility, presumably sufficient to permit the Agency to make a reasoned evaluation of the health and environmental effects of the microorganism under the conditions of use. Future TERAs built on the framework would allow EPA to streamline and focus its future TERA reviews more narrowly on the relevant properties of the organism rather than the sufficiency of baseline information or control and monitoring techniques. With greater experience, EPA will need to develop a practicable decision framework for the research program TERAs anticipated by the regulation, balancing innovators' need for nimble flexibility during R&D with a reasonable and appropriate level of environmental protection consistent with TSCA. We hope EPA will review this TERA with these larger goals in mind.

We have undertaken a scientifically rigorous process to establish:

- An environmental genomic "baseline" for SGI's California Advanced Algae Facility (CAAF) site and surrounding areas and ongoing monitoring program to assist in monitoring whether the engineered test strains could cause detrimental effects at a small scale in microbial communities and local ecosystems;
- Validated molecular and microbiological methods to characterize both wild-type and engineered algae growth characteristics in samples taken from local waters and soils;
- Validated analytical methods and monitoring instrumentation to aid in the development of a robust risk assessment process that can be used by academia and industry; and
- Best Practices for the responsible selection, handling, cultivation, processing and testing of engineered microorganisms.

SGI and ExxonMobil's ultimate goal is to develop renewable, sustainable, low-carbon, biofuels at world-scale volumes. The research permitted by this TERA is critical to our efforts to reach this goal.

## 4) Description, Uses, and Cultivation

### I. What are microalgae?

Single-celled ***micro***algae (not to be confused with plant-like ***macro***algae or seaweeds) are found virtually everywhere – in the Arctic ice, in every desert, ocean, and lake. Microalgae contribute a major share of global primary productivity forming the base of the global food web. They come in a huge variety of shapes and colors and can grow in cold and very hot climates, and in

both salt and freshwaters (**Figure INT 1**). They are photosynthetic – converting the energy in sunlight into chemical energy within their chloroplasts – and use this energy to grow, reproduce, and then store excess chemical energy in fatty-acid oils, starches and other biomolecules. The photosynthetic process simultaneously fixes atmospheric $CO_2$ into biomass while producing oxygen ($O_2$).[3] The energy-dense algae oils which are produced can be harvested and directly used to produce food and feed or can be converted in a refinery to biofuel. Until 1998 the U. S. Department of Energy maintained its own program to develop algal biofuels,[4] which it used to shape the National Algal Biofuels Technology Roadmap.[5]

Photosynthetic microalgae are frequently used to directly produce food and nutritional supplements, such as *Chlorella* and *Arthrospira platensis* (*Spirulina*). One of our neighbors in the Imperial Valley, Earthrise Nutritional, produces both the nutritional supplement *Spirulina* and a brilliant-blue food color extract derived from it.[f]

SGI has also applied its broad microalgae expertise to discover and license to ADM an Omega-3 fish oil replacement[g] derived from the heterotrophic microalgae *Aurantiochytrium* (heterotrophs use carbohydrates as an energy source instead of sunlight).

## II. Research and Development Process

Production of microalgae begins in the laboratory. **Figure INT 2** shows how individual cells are isolated from environmental sampling expeditions (also called "bioprospecting") and from global private and public culture collections. Step-by-step, a single cell is nurtured to grow and divide in small test tubes with growth media (nutrients, fertilizer, trace minerals), grown in increasingly larger quantities in small flasks, transferred to larger flasks, and when a culture is grown to sufficient size inside enclosed large photobioreactors (sunlight-transparent re-circulating tubes and/or bags), it is pumped into large ponds. These outdoor cultures are then grown under very specific conditions, and once certain criteria are met, the biomass can be separated from the water via flocculation, gravity settling, filtration, centrifugation or some other method. The biomass then can be shipped for downstream processing and conversion into fuels such as jet fuel and biodiesel.

## III. SGI's Headquarters and California Advanced Algae Facility (CAAF)

SGI has considerable expertise in global bioprospecting, genomics and metagenomics, cell engineering, and algae cultivation (**Figure INT 3**). It has two climatically diverse locations – its corporate headquarters and genomics R&D facility sited in coastal La Jolla (San Diego), California, and its 75-acre aquaculture research station is sited 100 miles east, in the low desert of the Imperial Valley, near the shores of the Salton Sea in Calipatria, CA (**Figure INT 4**).

The CAAF (**Figure INT 5**) is a fully-functional aquaculture operation (aquaculture is classified as a type of agriculture). Its location is well-suited for algae testing and scale-up – plentiful sunlight, distinct seasons (extremely hot in the late spring through fall, and very pleasant during the winter), access to water, $CO_2$, and adequate infrastructure. Imperial County has one of the

[f] http://earthrise.com/linablue/what-is-linablue/

[g] https://www.adm.com/products-services/food/onavita

greatest concentrations of renewable energy industries in the U.S. – solar photovoltaic, small hydroelectric, wind, geothermal, biomass, and now, algae biofuels.[h]

Equipment and infrastructure are already in-place to support algae research. The site has a state-of-the-art water treatment and recycling facility, labs, storage space, utilities, etc. Water rights, permits and zoning are all supportive of SGI's current and anticipated activities.

The CAAF until very recently was also a USP-certified Good Manufacturing Practices (GMP)[i] algae-based nutritional supplement manufacturing facility, with excellent operational discipline and a strong safety record. Its diverse and professional culture is such that processes and procedures are well defined and strictly adhered-to.

## 5) Risk Management and Responsible Oversight

### I. Management of Risk

There have been several research articles over the past twenty years that have attempted to address the complex risk assessment process for outdoor field testing of engineered microorganisms – to include both bacteria and microalgae.[6-14] Consensus of opinion on the types of studies and data required to form a comprehensive risk assessment have recently begun to converge in EPA's 2016 *Draft Algae Guidance for the Preparation of TSCA Biotechnology Submissions.*[15] The environmental non-governmental organizations (ENGOs) Friends of the Earth[16] and Biofuels Watch[17] have written thorough whitepapers advocating for an approach outlined in EPA's draft guidance.

EPA's regulatory oversight of field testing also goes hand-in-hand with the NIH Guidelines which are discussed in the next section. Also highly relevant are the corresponding guidelines for wild-type and classically-improved microorganisms found in the US Centers for Disease Control and Prevention's (CDC) *Biosafety in Microbiological and Biomedical Laboratories*[18] manual (BMBL). Further practical guidance is found in Adair *et al. A Practical Guide to Containment: Plant Biosafety in Research Greenhouses.*[19]

### II. NIH Guidelines

SGI subscribes to the internationally-recognized US National Institutes of Health (NIH) *Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules*[20] (NIH rDNA Guidelines). They detail the safety practices and containment procedures for basic and clinical research involving recombinant or synthetic nucleic acid molecules, including the creation and use of organisms and viruses containing recombinant or synthetic nucleic acid molecules.[j]

### III. SGI's NIH Institutional Biosafety Committee

In order to conform to the NIH guidelines, all recombinant DNA (rDNA) programs are overseen and must be approved by SGI's NIH Institutional Biosafety Committee (IBC). The current IBC is

[h] https://www.drecp.org/counties/factsheets/Imperial_county.pdf

[i] 21 C.F.R. § 111. Current Good Manufacturing Practice in Manufacturing, Packaging, Labeling, or Holding Operations for Dietary Supplements

[j] https://osp.od.nih.gov/biotechnology/nih-guidelines/

composed of three external community members and two SGI compliance and safety employees not having a reporting relationship through the executive heading the research program. Occasionally, senior executives, and senior scientists from other programs, may lend their expertise to the Committee on an *ad hoc* basis. Special deference is given to the external community members on any questions or concerns they may have, and their concerns must be resolved prior to the IBC approving a project. IBC community members typically participate on the committee for several years.

The IBC reviews and approves all projects on a yearly basis, and its deliberations consider the local communities and our research staff. The IBC oversees and specifically approves engineering and procedural controls, biosafety level, PPE requirements, labs, greenhouses, and the configuration and use of other facilities such as the CAAF.

The current SGI biosafety committee members have the following backgrounds:

- President of a local community college
- Retired head of a local medical research institute's Environment, Health and Safety program (internationally-recognized expert)
- Retired algal biotechnology scientist/engineer from a local university's aquatic research institute (internationally-recognized expert)
- Manager, SGI Industrial Hygiene and Safety – reporting into Facilities & HR
- IBC Chair - Senior Director, SGI Regulatory Affairs and Quality – reports to CEO

## IV. Community Engagement

SGI had made sustained efforts to engage and establish positive, constructive relationships with the local community and as well as stakeholders at the state and federal level. We have met with local, state, and federal government elected and appointed officials, maintain educational engagements with high-school and community college STEM programs, local NGOs, business organizations, and have presented SGI technologies at numerous national scientific and local renewable energy conferences. SGI has also actively supported the development of federal guidance and the regulation of the rapidly advancing fields of synthetic biology and biosafety.[21-29]

## V. Organization of TERA Application Dossier

This dossier follows the organization, numbering and content of EPA's *Draft Algae Guidance*[15] *for the Preparation of TSCA Biotechnology Submissions* (with minor contextual adjustments).

# A. Recipient Microorganism Characterization

## 1) Taxonomy

This TSCA Experimental Release application (TERA) is for an engineered-derivative of a proprietary *Parachlorella* sp. strain. The wild-type *Parachlorella* (STR00010) from which the notified strain is derived was isolated from seawater samples collected by SGI near the Hawaiian island of Oahu, in accordance with federal and state regulations. The wild-type strain was subjected to classical strain improvement methods, specifically UV mutagenesis, which produced a classically-improved strain (identified as STR00012) with higher biomass productivity than the wild-type strain. Strain STR00012 is the recipient strain which is further engineered to yield the subject microorganism of this TERA application.

As part of SGI's safety, health, and environmental (SH&E) risk assessment process, it commissioned Nerac, Inc. in 2010 to perform a SH&E literature review for *Chlorella.*[30] The *Chlorella* review encompassed the broader *Chlorellaceae* family and as such included *Parachlorella* within it. SGI routinely commissions genus-level SH&E reviews in the course of evaluating new research candidates for advancement in its R&D pipeline. These reports are initiated early in the development process to facilitate the exclusion of candidates which might evidence an unacceptable environmental or safety risk.

Every SGI SH&E literature review includes an up-front and detailed evaluation of the pertinent current taxonomic scheme. This is of critical importance where there have been substantial changes. Taxonomic scheme evaluations can be tedious and intensive, but they have gained in importance, particularly with recent technological advances using nucleic acid sequencing technology. Having rapidly evolved from sequencing technology developed since the sequencing of the human genome,[31] nucleic acid sequence comparisons of small subunit ribosomal RNA (SSU rRNA) now provide a fundamental basis for rapid, detailed and accurate comparisons among and between organisms. The EPA in their 2016 draft algae guidance also noted that modern classification schemes for microorganisms now rely primarily on nucleic acid sequence analyses.[15]

Renaming of taxonomic units – especially genera – does occur, so it is necessary to determine which references using older taxonomic names are applicable to the genera and species of interest. Then, relevant documents published when the subject organism was classified under an older name should be located and included for consideration. Correspondingly, those reclassified or misclassified species that are removed from a genus of interest should be excluded from consideration.

In the years since the 2010 *Chlorella* SH&E report, the taxonomy of the *Chlorellaceae* family has undergone further refinement. As a consequence, SGI commissioned Select Bio Consult Inc. to perform a SH&E literature review to specifically address the genus *Parachlorella.*[32] This report contains a summary of the literature including citations used directly in the review, and a list of all other citations found, including the areas of taxonomy, geographic distribution, environmental interactions, and reports of the impact of the genus on the environment. Details from this report are integrated throughout this application.

As part of the process for confirming the correct taxonomic basis for STR00010, we used the nucleotide sequence of the nuclear 18S SSU rRNA, a common phylogenetic marker, to aid in substantiating our

strain as belonging to phylum Chlorophyta, class Trebouxiophyceae, order Chlorellales, family *Chlorellaceae*, genus *Parachlorella* (for nucleotide sequence see **Appendix A1**). To place STR00010 in the context of other known *Chlorella* strains, we created a phylogenetic tree based on the analysis of 18S rRNA gene sequences (**Figure A1**). We selected the 18S rRNA sequences that were previously included in the published analysis of the *Chlorella* NC64A 18S rRNA gene plus the top blast matches to STR00010 rRNA sequence (*Chlorella* strains KAS012, SAG211-18, MBIC10088). The phylogenetic grouping suggests that STR00010 is part of the *Parachlorella* clade and is divergent from the so-called "true *Chlorella*" clade.[33] While specific phylogenetic relationships continue to be refined, the genus *Parachlorella* was shown to be a sister phylogenetic clade closely related to the "true" spherical *Chlorella.*[34]

The phenotypic plasticity of the *Chlorellaceae* has resulted in much of the historical mis-classification of these organisms. As such, the classification of STR00010 strain relies strongly on nucleic acid analyses. Notwithstanding, *Parachlorella* STR00010 is phenotypically and morphologically consistent with a *Parachlorella* assignment. STR00010, and strains derived thereof, grow as a small (2-3 µm in diameter), unicellular, spherical cell (**Figure A2**).

## 2) General Description and Characterization

As discussed above, the recipient organism was isolated by SGI as part of a broad bioprospecting effort. Laboratory enrichment cultures were established employing various media and cultivation conditions. The subject organism arose in an enrichment culture consisting of an artificial seawater medium containing 50 ppt sea salts. The culture was maintained at 30 °C. As might be expected from a strain isolated from such sampling and enrichment conditions the recipient strain grows well in a variety of temperatures and salinities.

To better characterize the environmental conditions permissive to, as well as optimal for, growth of *Parachlorella* STR00012, growth rates were determined under varying environmental conditions. Abiotic conditions assessed for minimum, maximum, and optimum growth included temperature, salinity, pH and bicarbonate level. These tests employed small (~25 mL) cultures grown in vented bioreactor tubes on shaker platforms. Standard conditions were 25 °C, 35 ppt sea salts, pH 8.0, with no added bicarbonate. Standard conditions were employed for all of these parameters with the exception of the one variable being tested. Typically, the growth rate is calculated over a period of several days. In general, the growth rate under standard conditions is somewhat less than one doubling per day, ranging from approximately 0.5x to 1x. *Parachlorella* STR00012 is able to grow under a broad range of abiotic conditions with broad optima centered around 25 °C, 50 ppt salts, pH 7.5, with bicarbonate additions greater than 10 g/L further improving growth (**Figure A3**).

Recipient and subject *Parachlorella* strains grow as a uniform unicellular spherical cell. Alternate growth forms, such as filaments, colonies, spores, or cysts have never been observed with this strain. In general, *Parachlorella* reproduces asexually via mitosis. No flagella or sexual reproduction has been observed. Under standard nutrient replete growth conditions *Parachlorella* STR00012 is 66% protein (as amino acid), 18% carbohydrate, and 15% lipid (measured as fatty acid methyl esters, or FAMEs). The specific FAME profile of *Parachlorella* STR00012 is provided in **Figure A4**.

A detailed photophysiological comparison of the recipient and subject strain was completed to verify the absence of any photophysiological differences between the strains. Biological duplicate cultures

were acclimated to low light conditions prior to photo-phenotyping. Measurements were made of the maximum quantum yield of photochemistry in PSII (as $F_v/F_m$), functional absorption cross-section of PSII, light-saturated electron transport rate, $P_{max}$ by $^{14}C$ incorporation, as well as chlorophyll a (Chl a) and Chl b content of cells (**Table A1**). There was no significant difference between recipient and subject strains as for all measures the differences between strains were less than the error of the measurement (CVs typically less than 5%).

Both SH&E reviews detail the broad biogeographic distribution of *Chlorella* and *Parachlorella*.[30, 32] Our own bioprospecting efforts confirm this as well. SGI undertook a significant effort to isolate algae strains for our biofuels program, and in the course of this bioprospecting work, hundreds of *Chlorella*-like isolates were obtained. Specifically, we isolated 163 strains that are highly related (<1% sequence divergence of ~750bp of the 18S SSU sequence) to the subject microorganism of this TERA. These strains were isolated from samples collected in virtually every region we visited, including Hawaii, California, Pacific waters of Mexico, Caribbean Sea and Gulf of Mexico waters of Mexico, Puerto Rico, Florida, Texas, and even a saline spring in Utah. It is clear from a review of the literature,[32] as well as our own work, that *Parachlorella* is a globally distributed genus.

SGI has selected *Parachlorella* for this work for several reasons. At a base level, this strain is among the better biomass- and lipid-producing strains we have isolated during the early years of our biofuels program. In addition to growth characteristics, genome size, structure, and complexity were major considerations for our continued work with *Parachlorella*. *Parachlorella* STR00010 has a relatively small (~55Mbp) and simple haploid genome. Moreover, SGI has successfully developed the requisite toolbox for advanced genetic engineering of this strain. As such, we can generate strains with targeted genetic insertion. We regularly re-sequence engineered strains, which provides a high level of certainty of the final genetic construct and lack of off-target effects.

# B. Engineered Alga Characterization

## 1) Taxonomy of the TSCA Subject Microorganism

The taxonomy of the subject microorganism, hereafter *Parachlorella* (strain) STR26155, is identical to the recipient alga. This TSCA Experimental Release application is for an engineered-derivative of our proprietary *Parachlorella* STR00012 described in **Section A**. For this application we have generated an engineered strain with minimal intergeneric DNA. This strain was developed to have virtually no discernable phenotypic differences relative to the recipient (i.e. starting) strain, but which possesses a nucleic acid signature and corresponding reporter protein to allow us to specifically track this strain in open-culture and in the environment.

## 2) Taxonomy of the Donor Organisms/ Synthetic Sequences

The TERA strain has been engineered to express a green fluorescent protein (GFP). The expression of GFP has been characterized in many host systems and is heavily utilized as a reporter protein, which has minimal impact on the phenotype of the recipient organism. SGI selected GFP based on its well-understood characteristics and suitability as a means of identifying the bioengineered *Parachlorella* in environmental monitoring samples, whether by genetic analysis or microscopy, when cultivated in an open pond R&D setting.

A detailed description of the genetic modification to the subject engineered alga *Parachlorella* STR26155 is provided in **Section C**, below. There are two nucleotide strings that are not native to *Parachlorella*. The first is a short (34bp) loxP site which is derived from bacteriophage P1. We employed Cre-lox recombinase technology to remove the selectable marker as well as the Cre-recombinase itself to generate the subject strain. In doing so, a single loxP site remains in the subject genome. The second intergeneric nucleotide sequence is the green fluorescent protein "TurboGFP™" (Evrogen, Moscow, Russia). This fluorescent reporter protein is an improved variant of a green fluorescent protein originally isolated from a copepod[35] of the order Calanoida (a zooplankton found in ocean waters).

# C. Genetic Modifications

## 1) Construction of the TSCA Subject Microorganism

### I. Brief summary

For this TSCA experimental release application we have generated an engineered algal strain with minimal intergeneric DNA. This strain was developed to have virtually no discernable phenotypic differences relative to the recipient strain, but which possesses a nucleic acid signature and corresponding reporter protein to allow us to specifically track this strain in open-culture and in the environment.

A simplified flow-chart for the construction of the subject strain is provided as **Figure C1**. We have also published the development and implementation of these same methods in a different model alga[1]. At a high-level, the work can be broken down into four separate phases:

1) Plasmid construction by (SGI-developed) Gibson Assembly[36, k] of eight linear DNA fragments;
2) Co-transformation of construct into recipient strain (STR00012) with Cas9 RNP for targeted genomic recombination site (RS1);
3) Induction of Cre-recombinase for precise excision of unnecessary/unwanted construct elements, specifically the antibiotic resistance selection marker; and
4) Final verification of subject strain construction.

A detailed accounting of all genetic elements and the step-wise construction of the subject strain is provided below, a high-level summary is provided in this introduction to orient readers of this application. As numbered in the figure:

1) The genetic construct transformed into the recipient strain has three intergeneric genes, each with endogenous *Parachlorella* regulatory sequences. Additionally, the construct has several cloning elements such as restriction and linker sites to facilitate cloning and assembly. Importantly, the construct contains genetic elements and the coding sequence for Cre recombinase which, when expressed, can recombine the construct resulting in the self-excision of part of the construct. This approach was undertaken to allow us to remove the selectable marker in the final constructed strain;
2) The genetic construct was co-transformed into the recipient strain along with a Cas9 ribonucleoprotein (RNP) complex, which targets a specific genomic locus, which facilitates precision targeting to the desired genomic locus. Transformants were grown on selective media, PCR screened for intended insertion at the targeted genomic locus, and GFP expression confirmed;
3) With the desired clones in hand we induced the expression of Cre recombinase. Following induction, clones were once again PCR screened for intended excision of the

[k] https://www.sgidna.com/products/gibson-assembly-reagents/

selectable marker and Cre genes. Sensitivity to zeocin was verified, indicating loss of excised DNA; and

4) Lastly, the final construct was verified. This included, as labeled in the figure, (i.) PCR screening of the insertion site locus, (ii.) ddPCR screening to verify that the GFP gene is present as only a single copy, (iii.) Sanger sequencing of the insertion site locus and entire insert, and (iv.) whole genome re-sequencing to further verify insertion site as well as the lack of unintended off-target mutations.

## II. Detailed description of strain construction

*Plasmid construction by Gibson assembly of 8 linear DNA fragments*

Plasmid NAS14335 contains three intergeneric genes (Cre recombinase, CRE; bleomycin resistance protein, BLE; and a green fluorescent protein, GFP) that were identified from public databases and refactored for *Parachlorella* (**Figure C2** and **Table C1**). The Cre-recombinase enzyme binds to specific sequences called *lox* recognition sites and when these sites are in proximity to each other and share the same orientation, the DNA between the sites is excised and lost. The bleomycin resistance gene confers resistance to the antibiotic zeocin and is used as a method to select transformants. The GFP produces a fluorescent signal and is used as a reporter to differentiate the subject. CRE (NCBI accession P06956) was codon optimized for *Parachlorella* and synthesized with the first six introns from the recipient strain nitrite reductase (*NiR*) gene then cloned under the PCR-amplified (from the recipient strain) *NiR* promoter and terminator. The *NiR* promoter is induced in the presence of nitrate ($NO_3^-$) and repressed in the presence of ammonium ($NH_4^+$) so CRE expression could be controlled by modifications of the culture medium. BLE (NCBI accession Q6GKR3) was codon optimized for *Parachlorella* and synthesized with the first five introns from the recipient strain 40S ribosomal protein (*RPS4*) gene then cloned under the PCR-amplified (from the recipient strain) *RPS4* promoter and terminator. GFP was amplified from pTurboGFP-C and cloned under the PCR-amplified (from the recipient strain) promoter and terminator of the Acyl Carrier Protein (*ACP1*) gene.

The construct was assembled to place CRE and BLE between two 34 bp *loxP* sites. When repressed, lack of CRE expression allows the full cassette to be maintained stably, resulting in consistent BLE expression. This confers resistance to zeocin and an ability to select for transformants. Once induced, CRE expression causes self-excision of the DNA sequence between the two loxP sites. In this case resulting in the loss of the BLE and CRE genes. GFP was placed outside of the two *loxP* sites so it remained in the genome after Cre-lox recombination (**Figure C2** and **Figure C4**).

Plasmid NAS14335 was constructed through Gibson cloning of eight linear DNA fragments that came from either plasmid digests or as synthesized double-stranded linear nucleic acid fragments (“gBlocks™”, IDT), schematics and details provided and **Figure C3** and **Table C2** respectively. The three intergeneric genes were synthesized and cloned between PCR-amplified endogenous promoters and terminators in intermediate plasmids to make the fragments required for the final construct plasmid NAS14335.

Several small 25 bp non-coding synthetic linkers were also used as cloning elements to aid in the assembly of intermediate plasmids. Large homology regions (depicted in red in **Figure C3**) were used to stitch the fragments together using Gibson cloning methods. The final resulting plasmid was fully sequence confirmed by Sanger sequencing of the entire BAC insert.

**Table C3** provides a detailed accounting of all elements in plasmid NAS14335. Those intergeneric elements which remain in the final construct are highlighted.

*Targeted introduction of genetic construct into recipient strain*

The final plasmid NAS 14335 was prepared for electroporation by *PacI* digestion to release the integration cassette followed by spin column purification (Qiagen QIAquick™ PCR Purification Kit). Although the backbone was delivered to the recipient microorganism as a carryover from the spin purification, the backbone did not have the ability to self-replicate and sequencing of the subject microorganism confirmed the backbone did not integrate off-target in the genome (see re-sequencing details below). RNP components were all purchased from IDT and complexed before electroporation with the plasmid. CRIPSR RNA (crRNA) designed to target the RS1 locus was first annealed to the non-specific transactivating crRNA (tracrRNA) to make a single guide RNA (gRNA) that was then complexed with the CAS9 protein to form the active RNP.

After electroporation, the transformed cells were left to recover overnight in ammonium-rich media and plated onto $NH_4^+$/zeocin plates to repress CRE and select for BLE. Colonies that survived transformation were patched onto a secondary $NH_4^+$/zeocin plate to continue repressing CRE while validating plasmid integration and GFP expression.

Colony PCR was used to verify integration of the plasmid into the correct locus. Utilizing primer pairs which flank each side of the insertion site and into the insertion cassette. **Figure C4** provides a schematic of the locus and priming sites. **Figure C5** provides primer sequences and shows the PCR results (reactions 1 and 2) of the screening of twelve colonies. Of the twelve colonies screened, two clones had the appropriate insertion at the RS1 locus and clone #6 was used in subsequent strain construction steps to ultimately become STR26155.

Expression of the introduced GFP gene was confirmed by flow cytometry using an Accuri™ C6 cytometer (BD Biosciences). Cells from colony patches were resuspended in growth medium and verified to have GFP fluorescence using the emission filter appropriate for GFP fluorescence emission. All GFP lines showed clear shifts in the fluorescence of the cell population compared to wild-type control.

*Cre recombinase induction to specifically excise undesired DNA construct elements, specifically the antibiotic resistance selection marker.*

The Cre recombinase enzyme binds to specific sequences called *loxP* recognition sites and recombines them to excise the DNA between the sites when in the same orientation. Since the CRE is under the control of the *NiR* Promoter, it can be induced in the presence of nitrate ($NO_3^-$) and repressed in the presence of ammonium ($NH_4^+$).

Once an appropriate clone was validated at the RS1 locus by colony PCR, it was patched onto a plate containing a nitrate-rich medium to induce the *NiR* Promoter to begin expressing CRE. This initiated Cre-Lox Recombination at the *loxP* sites to excise the CRE and BLE genes between the sites, while leaving GFP at the RS1 locus (**Figure C4**). During this phase of strain construction media used did not contain zeocin as the BLE gene is intended to be lost and sensitivity to zeocin restored.

The patch was inoculated into nitrate-containing media to ensure CRE expression and after a series of passages in liquid culture cells were struck out on plates to isolate single colonies. Colony PCR was repeated which indicated a loss of the wild-type sized band and presence of a ~3 kb band, consistent loss of loxP flanked DNA and a single GFP gene insertion (**Figure C6**).

In addition to PCR screening, isolates were patched onto replicate agar plates with and without zeocin. Isolate 15 had a restored sensitivity to zeocin and became STR26155 (**Figure C7**).

*Final verification of construct*

PCR screening of the final strain has been presented above. These all indicate the proper insertion at the RS1 locus and subsequent loss of desired DNA by Cre recombinase activity. The full ~3 kb PCR fragment was subjected to Sanger sequencing to confirm the final construct down to the nucleotide level. The final strain STR26155 contains an intrageneric *HpaI* site and an intergeneric *loxP* site. It also possesses an intrageneric GFP gene with a paired endogenous promoter and terminator (from the *ACP1* gene) (**Figure C8**).

As is frequently observed in such targeted mutagenesis methods, which rely upon the efficient but error prone non-homologous end joining repair pathway, a small insertion is present at the site of insertion. For STR26155, a small (2bp) insertion is observed at the 3' end of the inserted gene cassette. No unaccounted-for nucleotides are observed at the 5'.

In addition to Sanger sequencing at the RS1 insertion site, droplet digital PCR (ddPCR) was performed with primers targeting the three intergeneric genes used in the strain's construction. This confirms the absence of CRE and BLE in the subject strain as well as validating that the remaining intergeneric GPF is present at a single copy. (**Figure C9**). GFP expression is also confirmed by epifluorescence microscopy and flow cytometric analyses of the subject strain.

## III. Final recipient strain characterization

*Prior modifications (deletions, additions):*

No prior modifications. As described above, the recipient strain STR00012 is a classically improved strain obtained through UV mutagenesis and subsequent strain screening. This classically improved strain (STR00012) has higher biomass productivity than the wild-type strain. STR00012 is the recipient strain for this TERA application.

*Presence of plasmids and their ability to promote mobility/transfer, or affect the expression, of the introduced genetic material:*

There is no evidence for the presence of plasmids in the recipient *Parachlorella* STR00012. PubMed literature searches for *Parachlorella* AND plasmid as well as *Parachlorella* AND mobil*, at the time of this application, return zero matches. Moreover, as part of our research program we commonly generate whole genome sequences as we re-sequence variant strains of interest. We have re-sequenced *Parachlorella* many tens of times and never observed extrachromosomal elements beyond the organellar genomes from the chloroplast and mitochondrion.

*Gene sequences and whole genomes where known*

SGI has a high-quality genome sequence for the wild-type *Parachlorella* STR00010. Additionally, results of whole-genome resequencing of subject strain STR26155 are discussed below and sequences relating to construction of and final construct are presented in **Appendices C1 and C2**.

*Stability of gene integration*

Several lines of evidence indicate that the gene integration performed in subject *Parachlorella* STR26155 will be stable. The first is the use of *Parachlorella* STR00010 (and its derivatives) by our research program for genetic engineering over the past five years. Over this time, we have generated many hundreds of strains with gene integration events. Once transformed, screened, and selected we rarely, if ever, observe instability of our genetic constructs. While explicit testing of genetic stability for a given strain/construct over many generations is rarely done, observational evidence from a significantly large strain engineering program suggests that genetic instability is low. Of particular note are two parental strains which have been used as the base strain for the construction of hundreds of daughter lines each over the years. These two lines each contain gene integration events themselves with elements important for efficient genetic engineering efforts.

The continued use of these two strains, over many years, provides evidence of the stability of gene integration in *Parachlorella*. A second line of evidence is more specific to the subject strain of this TERA. We have generated dozens of strains targeting gene integration to the RS1 site employed in this work. Again, continued use of parental strains, with integration at this specific locus, without genetic instability lends weight to the expectation of low genetic instability for the subject microorganism.

Lastly, experimental data from subject strain STR26155 also indicates low/no genetic instability. We have cultured STR26155 for an extended period with no sign of genetic instability. For approximately three months, STR26155 was semi-continuously cultured by weekly dilutions. Conservatively, this timeframe and culturing approach has put the strain through >120 generations. PCR screening of the culture after three months of propagation confirms the genetic insertion at the RS1 locus, with no indications of alternative forms.

*Location of endogenous gene(s) homologous to the introduced nucleic acid sequences that could promote mobility/transfer of the introduced genetic material*

Detailed in **Figure C3** and **Table C3**, we have used endogenous elements (specifically promoter-terminator or promoter-intron-terminator combinations) to control expression of intergeneric genes. The targeted genomic insertion site is in chromosome 6, while endogenous elements for cre, ble, and GFP expression are borrowed from chromosomes 7, 1, and 3, respectively. Inter-chromosomal recombination is not expected to occur between the native and re-purposed endogenous genetic elements.

*Characterization of the insertion site for the introduced genetic material*

The target insertion site (hereafter called Recombination Site 1 or RS1) was selected with the aid of both genome and transcriptome data. The site was chosen because it was a larger intergenic region with no detectable transcription. The goal was to minimize the chance of unintentionally disturbing the function or regulation of nearby endogenous genes due to our integration.

*Use of antibiotic resistance marker genes*

A bleomycin resistance gene, originally isolated from the actinomycete bacterium *Streptoalloteichus hindustanus*,[37] was used for clone selection at intermediate points of strain construction. This gene confers resistance to the bleomycin family of antibiotics. In our work we utilize zeocin, a formulation of phleomycin D1. This gene was removed by Cre recombinase and is no longer present in the subject strain.

*Characterization of gene silencing/RNAi technology employed and description of gene(s) that are downregulated*

Not applicable. Gene silencing/RNAi technology is not employed.

*Stability of gene silencing*

Not applicable. Gene silencing/RNAi technology is not employed.

*Potential for transfer of RNAi to non-target organisms*

Not applicable. Gene silencing/RNAi technology is not employed.

## IV. Plasmid maps

See **Figure C2**, and **Table C2**.

## V. Fragments

See **Figure C3**, and **Table C3**.

VI. Methods for isolating and identifying the nucleic acid sequences used to modify the recipient microorganism

SGI uses standard methods for isolation of nucleic acids. We typically employ commercially available DNA extraction and purification kits or standard phenol-chloroform DNA extraction methods. [l,m]

VII. Commercial systems

See **Table C4**.

VIII. Accession numbers

See **Table C1**.

## 2) Final Genetic Construct

**Figure C8** and **Table C5** provide a schematic and full details for the final genetic construct. The final strain, STR26155 contains, an introduced *HpaI* restriction site, an intergeneric *loxP* site, an intrageneric *ACP1* promoter, intergeneric GFP gene and intrageneric *ACP1* Terminator. These are all located, as targeted by CRISPR-induced double-strand break, at the desired RS1 locus. This final strain construction has been initially verified by Sanger sequencing. As expected (and as indicated by PCR screening), the introduced gene cassette is present at the RS1 locus. As is frequently observed in such targeted mutagenesis methods which rely upon the efficient but error prone non-homologous end joining repair pathway, a small insertion is present at the site of insertion. For STR26155, a small (2bp) insertion is observed at the 3' end of the inserted gene cassette. No unaccounted-for nucleotides are observed at the 5' end. (see **Appendix C2** and **Figure C8** for details). The RS1 site was specifically selected as a neutral region with no nearby coding sequences, hence this small difference is expected to be neutral.

The target locus was PCR amplified and Sanger sequenced as one level of final construct verification. Whole genome re-sequencing was conducted on subject strain STR26155 to further confirm the final genetic construct. While this is not a standard component of our strain engineering workflow, we undertook whole-genome re-sequencing as an extra level of strain verification. These data provide an extremely high level of certainty for the confirmation of the lack of off-target or unintended genome modifications. Genomic DNA from STR26155 as well as recipient strain STR00012 (for use as a control) were used to prepare Truseq™ PCR-free libraries for Illumina sequencing. Over 185 million paired-end reads were obtained for both samples (STR26155: 186,644,712 reads, STR00012: 275,182,286 reads). Reads were mapped to the STR00010 genome assembly using the Burrows-Wheeler Aligner (*BWA-mem*) program, with a mapping efficiency of 99.7% and 99.8%, respectively, resulting in a median genome coverage of 457 and 646, respectively. SNP and indel variants were called with *freebayes* and filtered for high-confidence variants (i.e., minimum depth of 100, minimum quality of 100, minimum allele bias p-value of 0.05, and either strand bias ratio less than 1.1 or strand bias p-value greater than 0.05). The resulting variants were annotated with the program *SnpEff*[38] to classify variants by region and effect. Four SNPs and 180 putative small indels were identified in STR00012 relative to STR00010.

[l] https://www.qiagen.com/us/shop/sample-technologies/dna/genomic-dna/
[m] https://en.wikipedia.org/wiki/Phenol%E2%80%93chloroform_extraction

The majority of these mutations fall within intergenic or intron regions and are predicted to have no or low phenotypic effect. Only two variants are predicted to affect splice junctions donor/acceptor sites. The same 184 variants are recapitulated in STR26155, with one additional SNP observed at position wt01185_meta1_scf7180422_q:13436, a gene free region.

Examination of STR26155 reads aligned to the STR00010 assembly provides additional evidence of the insertion site at RS1 locus. A read gap is visible at the insertion site due to STR26155 read pairs that overlap with the construct, thus not able to align to the reference (**Figure C10**). Note that no such gap was observed at the same region for STR00012 reads.

Including NAS14335 in a mapping analysis of STR26155 sequencing reads reveals no reads mapping to the construct backbone (**Figure C11**). This is strong evidence for the lack of unintended insertion of the vector backbone into the subject strain genome.

Alignment of STR26155 reads to a genome assembly consisting of STR00010 and the predicted RS1 locus after insertion again shows >500x coverage at the GFP gene including around the regions flanking 5’ and 3’ of the insertion site (**Figure C12**).

# D. Potential Human Health Effects of the Engineered Alga

## 1) Pathogenicity to Humans

### I. Wildtype and engineered organism

Detailed literature reviews commissioned by SGI for both the *Parachlorella* and *Chlorella* genera have not identified any reports citing pathogenicity of members of either genus.[30, 32] Infections caused by green algae, often referred to as "chlorellosis", have been described in humans and other mammals.[39] The causative agent associated with these infections are members of the genus *Prototheca*, which despite being classified as "green algae", have lost their chloroplasts and all photosynthetic ability. This genus is also a member of the family *Chlorellaceae* along with *Chlorella* and *Parachlorella*, and the term "chlorellosis" predates common molecular identification techniques that are currently used to separate morphologically similar organisms. While the term "protothecosis" has also been used in the literature in reference to such infections, the use "chlorellosis" persists and has been extended to describe infections caused by green algae that belong to taxonomic families other than *Chlorellaceae*.[40]

GFP is not associated with virulence or pathogenicity, and the addition of the GFP gene would not be expected to impact this aspect of *Parachlorella* physiology.

## 2) Toxin Production

### I. Wildtype and engineered organism

The wildtype strain is not known to produce any toxins, and GFP is not toxic nor associated with the production of any toxins. Detailed literature reviews commissioned by SGI for both the *Parachlorella* and *Chlorella* genera have not identified any reports citing toxin production.[17, 30, 32, 35]

### II. Toxicology studies of GFP

One toxicology study of weaned rats fed recombinant GFP produced by transgenic canola found that ingestion of GFP did not affect growth, food intake, relative weight of intestine or other organs, or activities of hepatic enzymes in serum, indicating that GFP is unlikely to represent a health risk in humans.[41]

## 3) Immunological Effects of the Engineered Alga or its Products

### I. Wildtype and engineered organism

It is highly unlikely that either the wildtype or the GFP-engineered strains will cause immunological effects in humans. No reports were found indicating allergic responses to *Parachlorella* or organisms in its clade, and no evidence of allergic reactions to the closely related genus *Chlorella* were found.[12, 30, 32]

### II. Introduced GFP sequence

Green fluorescent protein in nature is distributed globally[42] and is used in a great many scientific products. Addition of the GFP gene is highly unlikely to cause the engineered strain to induce an immunological response in humans. The GFP toxicology study referenced in the toxin

section above also included an amino acid sequence comparison against known food allergen sequences. It found there was an absence of common allergen epitopes, combined with the rapid degradation of GFP during simulated gastric digestion also indicates that GFP has a low allergenicity risk.[41]

The protein sequence encoded by the introduced GFP gene was used to query the Food Allergy Research and Resource Program "AllergenOnline" database[n] (V19, accessed February 10, 2019). We ran database queries using the full 233 amino acid sequence, a sliding 80mer window, as well as 8mers. No results above the database threshold (e-value <1, >35% identity, and 100% identity, respectively, **Appendix D1**) were returned, indicating an extremely low likelihood of allergenicity for the encoded GFP protein.

### 3) Harmful Volatile Compounds

#### I. Wildtype and engineered organism

It is highly unlikely that either the wildtype or the GFP-engineered strains will emit harmful volatile compounds, such as methane derivatives or volatile fatty acids. No reports were found indicating emission of harmful volatile compounds.[32]

#### II. Introduced GFP sequence

The introduced GFP is non-volatile and is intracellular (not excreted) from the notified organism. Most proteins in nature are non-volatile, remain in solution, and do not enter the gas phase.

### 4) Presence/Prevention of Microbial Pathogens (Contaminants) in Ponds

All water used in production systems passes through a multi-step filtration process. This includes both intake canal water as well as recycled production waters. All water is passed through a three stage multi-cap filtration system. Following this, water is further subjected to a five-stage, one-micron bead filtration system. These processes are to ensure the removal of all cellular material. Filtered water is stored in darkened vertical storage tanks for ozonation and prior to use in production systems.

Taking into account our use of NIH GLSP (Good Large-Scale Practices),[20] we are acutely interested in understanding and tracking the organisms that co-culture along with our production algae strains. We employ microscopy, flow cytometry, as well as nucleic acid-based methods for the identification and classification of these co-culturing microbes. As such, we have good insights into the inhabitants of our open production ponds and will periodically monitor for incidental co-culturing of pathogens.

[n] http://www.allergenonline.org/

# E. Potential Ecological Effects of the Engineered Alga

**Table E1** summarizes our qualitative ecological hazard assessment for engineered algae. In summary, we do not expect that the TERA strain could pose an unreasonable risk to the environment. There is no record of *Parachlorella* toxicity or pathogenicity in environmental receptors. In addition, the alterations intended for the TERA strain are not intended to increase ecological fitness. Indeed, they are designed to be neutral to the fitness of the engineered alga, and thus we do not expect the TERA strain to exhibit a competitive advantage in the natural environment.

In general, microalgae genera having no toxic or infectious character pose little hazard to ecological receptors; many ecological receptors rely on microalgae for food or for the cycling of nutrients. Some variation in population density and composition can occur when a bloom of one species occurs, even if that species does not produce toxins. Engineered microalgae, lacking DNA sequences coding for toxins or infectivity, would not be expected to represent an elevated toxicity or pathogenicity risk. The potential exists for unanticipated impacts on population-level interspecies competition, and local biogeochemistry. However, strain characterization data presented above in **Section A** as well as growth and competition experiments presented below in **Section F** both suggest the subject strain will behave similarly to the recipient strain.

## 1) Toxicity to Animals

It is highly unlikely that either the wildtype or the GFP-engineered strains are toxic to animals. Detailed literature reviews commissioned by SGI for both the *Parachlorella* and *Chlorella* genera have not identified any reports citing toxicity.[30, 32] As detailed in Section D.2, no reports of toxin production by *Chlorella* or *Parachlorella* have been identified in the literature and the GFP construct contains no sequences associated with toxin production. It is therefore highly unlikely that the addition of the GFP gene would alter the lack of toxicity of *Parachlorella*.

Microalgae species are ubiquitous in the environment and can be found in aquatic, terrestrial, and aerial environments. Wildlife of all taxa are exposed to naturally-occurring microalgae in their environment due to the presence of the algae in water, air and soil. Some microalgae may be commensal or epiphytic on both terrestrial and aquatic plants and animals and on man-made surfaces.[43-45] Aside from toxin-producing species which can cause broad ecological effects, many aquatic microalgae species are at, or near, the bottom of the food web, and provide nutrition to primary consumers. Their suitability and value as food sources for protein and lipids are characteristics which have been exploited in aquaculture and agriculture, as noted in earlier sections of this application. In addition, they are the base of the food chain for many aquatic ecosystems in streams and small rivers, where ecologically sensitive and important species reside. Alterations in algal community composition or function are, in fact, often used as a measure of ecological health.[46-47] The following discussion is focused on aquatic algal species except where information from terrestrial or aerophilic algae are informative.

Microalgae serve as the base of the food web in aquatic ecosystems, as primary producers fixing energy from the sun into lipids, proteins, and carbohydrates which are then used by primary consumers to build their own biomass. Some animal species such as hydroids (corals) and mollusks (sea slugs) have a commensal relationship in which they provide a microhabitat for photosynthetic microalgae within their tissues and then make direct use of the photosynthetic products for their own

metabolism (that is, the host organism does not need to forage for dietary algae and does not consume the algae it hosts). Terrestrial animals are exposed to microalgae incidentally in food, air and water and are typically unaffected by them. Water and shore birds do not appear to have an increased incidence of harm from exposure to algae, compared to less-exposed species or types of wildlife. We would expect this to be no different for water and shore birds around the Salton Sea.

### 2) Pathogenicity to Animals

As discussed in **Section D.1.I**, while systemic infection by green algae has been described in humans and animals, it is highly unlikely that either the wildtype or the GFP-engineered strains are pathogenic to animals. Detailed literature reviews commissioned by SGI for both the *Parachlorella* and *Chlorella* genera have not identified any reports citing pathogenicity to animals.[30, 32] It is highly unlikely that the addition of the GFP gene would alter this aspect of *Parachlorella* physiology.

### 3) Pathogenicity to Plants

Algae parasitic to land plants are known only among a very limited number of green algae genera, specifically the *Chlorophyta* and *Chlorochytrium*, and instances of parasitism occur mainly on non-cultivated plants.[48] *Cephaleuros*, commonly known as "red rust", has been known to be parasitic on some important economic plants of the tropics and subtropics such as tea, coffee, mango and guava, causing damage limited to the area of algal growth on leaves (algal leaf spot), killing new shoots, or disfiguring fruit.[49]

There is no indication that wild-type or modified *Parachlorella* have or will exhibit pathogenicity to plants. The genus *Parachlorella* is native to the waters of California and broadly distributed throughout the U.S. SGI has isolated multiple wild-type species from the areas surrounding the CAAF and other Southern California locations, as well as from numerous other ocean and inland waters. Given its broad global distribution, combined with the literature reviews commissioned by SGI for both the *Parachlorella* and *Chlorella* genera which did not identify any reports citing pathogenicity to plants,[30, 32] it is highly unlikely that wild-type *Parachlorella* is harmful to plants, including those agricultural crops cultivated in the Imperial Valley. It is also highly unlikely that the addition of the GFP gene would alter this aspect of *Parachlorella* physiology and induce pathogenicity to plants.

### 4) Propensity for Bloom Formation

No bloom reports, including those for harmful algal blooms (HABs), were found in a comprehensive literature review, despite the presence of *Parachlorella* in many natural water environments. Likewise, the closely related genus *Chlorella* does not appeared to be associated with algal blooms or HABs.[30, 32] It is highly unlikely that the addition of the GFP gene to *Parachlorella* will induce harmful algal blooms. While strain selection for this and future TERAs and MCANS will include consideration of bloom propensity, the current strain's inclusion of GFP is not anticipated to change the (lack of) potential to bloom.

Some microalgae species present within aquatic habitats are noted for forming blooms in response to favorable (for that species) environmental conditions such as high nutrient concentrations,[50-51] warm temperatures,[52-53] or a change in salinity.[54] During those intervals, high densities of a single species occur which can have both benefits (in terms of providing a food source for predators and spurring their growth) and drawbacks, as the aquatic environment experiences rapid changes in sunlight and

oxygen availability due to the algal bloom. The blooms are typically only hazardous if toxin-producing species are responsible for the bloom, although secondary effects due to the proliferation of bacteria or viruses may also occur. Blooms are typically of limited duration within the window of optimal conditions. Although blooms are a natural occurrence, their location, frequency and severity may be altered by human activity including introduction of excess nitrogen and phosphorus into aquatic habitats, altering the hydrology of waterways resulting in increased salinity or movement of the salt line, and other habitat alterations.

The growth rate of the engineered strain will be documented as part of the outdoor testing, to be compared to wild-type growth rate. In addition to this general discussion, **Section F** details several experiments which indicate that the subject strain has no greater propensity for bloom formation than the recipient stain. Tests of growth in sterile-filtered CAAF-area waters (**Figure F3**) as well as competition-type experiments (**Figures F5 - F8**) all indicate equivalent growth from recipient and subject strains.

### 5) Potential Effects on Primary Productivity

**Section F** details several experiments which indicate that the subject strain has no greater propensity to impact primary productivity than the recipient stain. Tests of growth in sterile-filtered CAAF-area waters (**Figure F3**) as well as competition-type experiments (**Figure F5, Figure F7** and **Figure F8**) all indicate equivalent growth from recipient and subject strains.

### 6) Potential Effects on Other Biogeochemical Cycles

While the GFP-algae are not engineered to provide advantages in nutrient or other resource usage, they also are not intended to be less environmentally fit. Laboratory and greenhouse studies have demonstrated similar growth to wild-type parent strains.

Microalgae, particularly diatoms, are the base of many food chains/webs and are responsible for a significant portion of the world's primary and hence microalgae are major contributors to the earth's carbon cycle, in addition to being major participants in the nitrogen and in various mineral cycles, such as silicon, sulfur, and metals including zinc, copper, manganese and magnesium.[43] Estimates of the total carbon fixed by microalgae as well as the degree of contribution of microalgae to oxygen production vary, although the key role of microalgae in both processes is not in doubt. As part of the carbon cycle, carbon is often sequestered in the form of carbonate by the action of microalgae. The effects of microalgae on the balance of carbon in the oceans and in the atmosphere include the lowering of the $CO_2$ concentration near the ocean surface resulting from algal carbon ($CO_2$) fixation.[55] Approximately 25% of the carbon from the upper ocean sinks into the ocean interior where it concentrates and acts to raise the carbon concentration of the oceans at the expense of atmospheric carbon. This is sometimes referred to as a biological carbon sink serving to reduce atmospheric carbon with microalgae playing a key role.

Linked with the carbon cycle are the nitrogen and phosphorous cycles. Both elements are required for the biosynthesis of essential molecules, including nucleic acids and proteins. Ongoing considerations of which of the two elements has the greatest influence on primary production, i.e. microalgae as a primary food source for other life forms, is not resolved, although it is clear that the concentrations of both profoundly affect algal growth.[56] Particularly, the ratio of nitrogen to phosphorus is important in

regulating algal growth.[57] Microalgae also contribute to the cycling of many other nutrients such as zinc, copper, manganese, and magnesium.

## 7) Potential Effects on Microbial Food, Other Ecologically Important Relationships, and the Surrounding Environment

Microalgae are globally distributed and are well documented in the Salton Sea.[58] The wide variety of distribution mechanisms and environmental conditions lead to varying assemblages in different habitats, but common algal taxa are broadly distributed among aquatic habitats possessing water quality and habitat structure in the range easily exploited by a species or genus. Blooms of microalgae occur because of changes in water quality, allowing proliferation of opportunistic species already present within the algal community. Blooms occur much less often by introduction of a foreign species into a habitat. Thus, invasiveness is not a trait typically discussed with respect to microalgae (or other free-living microbes with which they share these traits). As described in the Environmental Exposure Assessment below, the aquatic ecosystems near to CAAF are host to a variety of sensitive receptors, some of which are predators/consumers of the algae. In particular, barnacles (*Balanus amphitrite*) are noted to be prevalent within the Salton Sea.

Microalgae coexist in many habitats alongside other algae and vascular plants. Macroalgae and higher plants serve as a substratum for periphyton (attached microalgae) in many ecosystems.[44, 59] A search of available online resources (including Wiley Online, SpringerLink, PubMed, and Google), indicates non-toxic algal taxa are not documented to cause deleterious effects on other plant species, either by competition or direct action (e.g., infection) although competition for light between pelagic microalgae and rooted aquatic vegetation is apparent.[59] Blooms of nontoxic algae may decrease light penetration, resulting in slowed growth or death of some rooted, submerged aquatic plants (including kelp, which is a commercially important marine species). The propensity of the strain to form blooms under natural environmental conditions is not expected to be altered by the genetic modification.

The CAAF program is designed, in part, to establish a set of conditions optimal for the growth of the specific algal strain(s), both natural and engineered, that will produce bio-oil. While it is not the intent of the project to select or engineer microalgae that are able to tolerate a variety of environmental conditions, it is recognized that the strains may tolerate broader conditions than are present in the project system. Growth of both the recipient and subject strains have been examined in the presence of local competitor species (see **Section F**). The available literature on ecological competition and on the relative success of engineered in wild habitats also provide the basis for further assessment.

## 8) Bioaccumulation of Metals in the Microorganism, in Liquid and Solid Wastes, and in the Final Product from Flue Gas or Other Sources

A detailed literature review for the *Parachlorella* genus reports that the genus may accumulate heavy metals, herbicides and other toxic organic and inorganic compounds when the materials are present:

> *"However, in addition to accumulation, bioremediation by Parachlorella may occur via sequestration or metabolism. The accumulation of toxic materials may provide a way to pass the toxins through the food chain, but no cases of such food-chain related events were found in the literature. Moreover, consideration of the results appears to be most accurately interpreted as a reaction of the microalgae to their environmental conditions. No evidence was found of Parachlorella as causative in generation of toxins or harmful materials. Practical application of*

> *Parachlorella environmental properties is most often considered as potentially beneficial rather than threatening. Also, as noted in citations above, many species of microalgae have been shown to accumulate heavy metals and herbicides, so Parachlorella does not appear to present a unique hazard in this regard."*[32]

It is highly unlikely that the addition of the GFP gene will modify this uptake behavior.

# F. Fate of the Engineered Alga

In the context of ecological risk assessment (ERA), environmental fate relates to the persistence of the microalgae in the environment, while transport addresses the route of the microalgae from the source (CAAF raceway ponds) to the environmental media and thus to ecological receptors. A more detailed assessment is presented below in **Section I** which combines a characterization of environmental fate with anticipated modes of transport to produce a qualitative risk assessment for exposure to the environment.

There are substantial mitigating circumstances to the environmental release of any engineered or wild-type aquacultured microalgal species. In general it's not an easy existence for microalgae, since they are the base of the global food web, and as such a great many different organisms are their natural predators, including protists, rotifers, crustacea such as *Daphnia* and copepods, and fish.[43, 60] Predators of algae may be present at very low levels in the environment and not normally apparent, but depending upon the growth conditions and prevalence of prey, they may rapidly reproduce and overtake concentrated microalgae populations such as an algal bloom in a lake or a commercial aquaculture pond.[61] Furthermore, there are also aquatic viruses[62] and fungi[63] that can kill algae very quickly when exposed to the natural environment, including viral pathogens known to infect *Chlorella*.[64]

Until 2017 the CAAF was producing the food supplement astaxanthin (a deep-red natural antioxidant) extracted from wild-type *Haematococcus pluvialis*. During this time there were several instances where a chytrid fungi wiped out an entire one-acre, one-million-liter pond overnight. In our experience, it will be more likely that the CAAF operations will have greater difficulty keeping predators and disease out of the ponds than keeping the desired species in.

## 1) Fate of introduced algae (wild-type or engineered)

*Parachlorella* species are native to the Waters of the State of California and are an approved genus for the CAAF in its CA Aquaculture Registration (**Figure F1**). SGI has documented its presence in Southern California and specifically in the Imperial Valley. The fate of algal cells introduced into the surrounding environment is not expected to differ with respect to general environmental-fate processes. Only algal species registered via the CA Aquaculture Registration process[o] are used for outdoor cultivation. California Department of Fish and Wildlife (CA F&W) has previously registered individual species for possession and/or cultivation in the state. SGI has worked closely with CA F&W and they have reviewed and registered 23 separate *genera*. Core to the risk assessment process are the SH&E Reports we commission for each genus that we plan to cultivate outdoors.

## 2) General ecological fate characteristics

Ecological fate characteristics are expected to apply to both classically-improved strains and engineered strains of the same host organism. The persistence of aquatic microalgae in the environment is dependent on survival of algal cells in environmental media. Aerobiology scholars generally accept the concept that airborne cells will die without a protective cover of the plasma membrane or the cellular wall, i.e., if they are not in a sporulated state.[65]

[o] https://www.wildlife.ca.gov/aquaculture

Species which are regarded as air or soil dwelling microalgae are typically specially adapted to withstand drying, UV exposure, and other environmental conditions to which aquatic microalgae are not resistant. Agrawal[66] evaluated viability of green and blue-green algal cells collected from aquatic and terrestrial media under various conditions. Following drying, water stress, heat stress, and UV exposure, they found short survival times in the aquatic green microalgae of one to six hours upon placement in a low-humidity environment (desiccator) or water-limited (with agar), and poor survival under relatively low UV doses and temperatures of 40 °C and higher. The blue-green microalgae, particularly those collected from buildings and dry rocks, were somewhat more tolerant of the experimental variables, with filamentous species appearing hardiest. Aquatic species (without resting stages) also appear to be less well adapted for atmospheric survival than aerophytic species.[67] Resting stages (cysts or spores) are typically produced over a period of time in which environmental cues indicate a need for resistance; conversely, conditions tailored for maximal cell and population growth will be used in the CAAF research program so algal cells which are released to air, or soil are expected to be in the least resistant, vegetative growth stages. Experiments designed to address the desiccation tolerance and viability of the recipient and subject are presented below.

A further difficulty for a microorganism to overcome in becoming established in an environment is that a small inoculum such as a droplet, or wash-off from a bird's feet or feathers, would generally readily be out-competed by native algae or eliminated via other environmental factors (e.g. stochastic effects).[68-69] In studies using phytoplankton, it was found that either an infrequent but large inoculum, or a more-frequent but small inoculum, could result in establishment of a population.[70] By comparison, a small, infrequent inoculum was far less likely to result in establishment of a population. While aquatic microalgae cannot be considered invasive due to their broad distribution, introduction of an engineered strain would be expected to follow the population dynamics described above.

### 3) Fate of engineered algae

There is extremely limited literature regarding the ecological behavior of engineered algae. To our knowledge, there is but a single published study[13] reporting the results from one microalgal TERA.[71] In their work, Szyjka *et al.* observed their subject alga in trap ponds, but only ever observed persistence in the algae traps closest to the experimental pond, immediately downwind of prevailing winds. Moreover, their greenhouse-based microcosm experiments which tested persistence and impact of the engineered alga on waters collected from five San Diego, CA water bodies showed no difference between recipient strain and subject stain treatments, with neither being able to outcompete local flora.

There is however, some information in the crop plant literature that may provide some insight. In general, directed variations from wild type have been found to not to be deleterious, or at least provide an adaptive disadvantage to present environmental conditions, so that a small release of an engineered plant (propagule) into the natural environment would not tend to persist – nor would the genetic modification, unless it became an adaptive advantage for a particular species.[72]

Crawley[73] found no evidence engineered oilseed rape was more persistent than conventional OSR in the wild; when there were significant differences (e.g., in buried seed survival) between the wild type and the engineered, the engineered was the poorer performer. In a further study of the invasiveness and persistence of engineered crops (oilseed rape, potato, maize and sugar beet), the engineered plants were found to be no more invasive nor persistent than their conventional counterparts, when

placed in a variety of different non-crop habitats for several years.[74] Another study revealed some engineered crop plant "volunteers" appeared in crop areas ten years after the annual crops were planted experimentally in the same plots and subsequently other crops were grown.[75] The volunteer plants were found at a very low rate (0.01 plants/$m^2$).

These findings highlight the expectation that engineered organisms may persist, and be detected in the environment, but persist at a low rate without exerting undue competitive pressure on the ecological community. The next section addresses how SGI will experimentally evaluate persistence.

### 4) Experimental assessment of recipient and subject algal strains

In order to assess the potential for survival and propagation in the environment we have tested, in the La Jolla greenhouse, the ability for both the recipient and subject organism to grow in waters collected from around the vicinity of CAAF (**Figure F2** and **Table F1**). We have identified nine sites within an approximate six-mile radius of our facility that represent various local aquatic biomes. Two sites, the Alamo river (IVF017) and an Imperial Irrigation District (IID) canal that supply irrigation water to our site (IVF004), are lotic environments which possess rapidly flowing freshwater year-round. Three sites are additional freshwater sites but more lentic with little or slow water flow. These include Morton Bay (IVF005, a marshland on the border of the Salton Sea), neighboring ponds maintained for waterfowl and fishing (IVF012), and the intake pond at CAAF (IVF001) which stores the canal water that feeds our site. Two sites represent emergent wetlands (IVF016) and seasonal riparian habitat (IVF010). Lastly, two sites provide samples collected from the Salton Sea proper (IVF006 and IVF008) which is moderately hypersaline at approximately 55 ppt salts. For the various experiments described below we have used water samples collected from these sampling stations. In addition, we have been visiting these sites monthly for over a year now to collect samples for microbiome analyses to provide a baseline for characterization of the local environment. We will continue to visit these sites monthly to further this environmental baseline and to serve as monitoring sites during and after the TERA experiments are conducted. This characterization and monitoring work are further detailed in **Section J** below.

SGI obtained samples from the described locations after we secured easements or permits from the Imperial Irrigation District (IID), the County of Imperial, and other contractual agreements with local private landowners and leaseholders, in compliance with local and state laws and the Convention on Biological Diversity[p]. These agreements grant access to land and water properties to collect samples and place algae traps.

### 5) Survival in Potential Aquatic and Terrestrial Receiving Environments

In order to assess the potential for survival and propagation in the environment we have tested, in the La Jolla greenhouse, the ability for both the recipient and subject organism to grow in waters collected from around the vicinity of CAAF (details above and in **Figure F2** and **Table F1**). We sterile filter (0.2 µm filter) local waters and then subsequently inoculated and tracked algal growth (by OD) over the course of approximately one to two weeks. Strains were grown in 50 mL vented bioreactor tubes on a shaker platform in our La Jolla greenhouse. Prior to the construction of the subject strain, we conducted this type of experiment using the wildtype strain STR00010 and the recipient strain STR00012. Each strain

[p] https://www.cbd.int/

was inoculated into the filter-sterilized site water at three inoculation densities (ODs 0.0005, 0.005, and 0.05) to ensure a minimum inoculum and to assess the importance of inoculum amount.

In general, both strains grew similarly in each local water type tested, although there were clear differences between the different water samples (**Figure F3**). Among the water samples water from the IID irrigation canal (IVF004) supported much higher growth than all other sample (generally supporting only a one- to two-fold increase in population in the other samples).

An identical preliminary experiment was conducted using water samples collected May 30, 2018 with results similar to those presented for water samples collected June 26, 2018. Similarly, some limited growth was observed in all waters tested. In this preliminary experiment however, waters collected from the input canal (IVF004) did not support the vigorous growth observed in June while the water from the neighboring duck/bass ponds (IVF012) and from the IID managed marsh (IVF016) did.

We conducted a repeat experiment using the subject and recipient strains. Similar to prior experiments there were strong differences by water type, in this case Alamo river water (IVF017) supported significant growth with most other waters supporting a doubling of growth or less (**Figure F4**).

Perhaps unsurprisingly, across all experiments, when the inoculation density was lowest, results were somewhat erratic; a minimum inoculation density (particularly for algae) can be required for consistent growth of cultures.

In addition to testing the survival potential of our *Parachlorella* strains in nearby aquatic receiving environments, a study was conducted to assess their desiccation tolerance. Soil from the CAAF site was collected and autoclaved to provide a sterile medium for the desiccation study. Many aliquots of soil (~1 g) were prepared in sterile 100 µm sieves which were spiked with several hundred µl of dense culture to deliver approximately $4x10^7$ cells onto the soil surface. Immediately at $T_0$, and for each subsequent timepoint, triplicate sieves for each strain were sacrificially sampled. To recover cells, 2 mL of culture medium was applied to sieves and subjected to gentle centrifugation. This procedure was repeated a second time with another 2 mL media. The resulting eluate was serially diluted and spread on agar plates for subsequent counts of colony forming units. Soil samples were maintained out of direct sunlight but subject to the ambient conditions of our greenhouse located in La Jolla, CA. Presumably the temperature, humidity, and light conditions at CAAF would be substantially harsher than the conditions used in this experiment which thus provides a conservative estimate for the desiccation tolerance of our strains in the Imperial Valley. A precipitous drop in viability is observed for both strains resulting in a 4 to 5 order-of-magnitude reduction in viable cells in the first week (**Figure F5**). Viable cells were detected at low levels after three and seven weeks. A final data point was collected after twelve weeks of incubation and nearly no viable cells were detected, as only one single colony was observed across the six samples.

## 6) Competition with Indigenous Species

The experiments described in **Section F.5** investigate the potential for our recipient and subject strains to survive and proliferate under the abiotic conditions present at the CAAF facility. We conducted a similar series of experiments to experimentally test the ability of the strains to survive, proliferate and potentially impact the environment in the presence of competition from the endogenous microbiota. While in the field, waters from selected stations were passed through a 106 µm stainless steel sieve to remove detritus, larger zooplankton and protists but were not manipulated further in an effort to

maintain the native microbiota. Once back at our La Jolla CA greenhouse, replicate 2 L vented flasks were prepared with ~1.25 L of these water samples. As above, prior to the construction of the subject strain, experiments were conducted using the wildtype strain STR00010 and the recipient strain STR00012. Triplicate flasks were prepared for each strain/water combination and included no-inoculation controls. Prior to the start of the experiment a pre-culture for each strain was grown in our greenhouse to ensure proper acclimation to the greenhouse conditions. For these experiments, from each preculture, an appropriate volume was used to inoculate experimental flasks to an OD of 0.005 for each strain. This inoculation density was shown to be sufficient for survival and growth in the abiotic condition. Flasks were incubated on shaker tables in the SGI greenhouse.

Over the course of approximately 3 weeks, samples were collected weekly for measuring growth (both as OD and total organic carbon (TOC)), as well as for microbiome profiling (methods described in greater detail in **Section J**, below). Each flask contained a complex microbial community and as might be expected OD data provided insights on general trends but was fairly erratic. More consistent were TOC data, which provides a measurement more independent of its cellular and potentially-heterogeneous composition. Different water sources possess varied levels of ambient TOC. While some minor differences are observed over the course of each experiment, generally the experimental treatments and uninoculated controls behaved very similarly (**Figure F6**). These results show that the introduction of recipient and subject strains has not grossly changed the productivity of the system, nor has a major algal bloom been produced.

Whole microbiome profiling was conducted on samples from this experiment as well. **Figure F7** present an NMDS (non-metric multi-dimensional scaling) plot which attempts to simplify the data and represent the similarity between samples in low-dimensional space. Each point represents a single sample and spots clustering close together indicates more similar whole microbiome profiles. All samples from flasks containing water from station IVF005 cluster very discretely (circled in blue) separated from samples from station IVF012 waters (circled in yellow). In both cases, a clear progression over time is observed (light grey arrows) yet controls and treatment flasks cluster very closely indicating highly similar microbial communities over time and at the end of the experiment. More significant differences are observed over the first few weeks but generally $T_2$ and $T_3$ cluster quite closely suggesting these experimental flasks may have reached a somewhat stable composition.

Once we had the subject strain constructed, we conducted a repeat experiment using the subject and recipient strains. Similar to prior experiments, there were strong differences by water type, but no significant differences in primary productivity of the microbial consortia between either strain or no-inoculation controls (**Figure F8**). Also similar to initial experiments, microbiome profiling produced a strong clustering primarily by station, secondarily by timepoint and almost no distinction between either strain or the no-inoculation control. These data can be queried more in depth to specifically track subject and recipient *Parachlorella* strains in these experimental flasks. Clearly both recipient and subject strain are able to establish themselves within the microbial community, and each represents the dominant microbe in the flasks at $T_1$ and $T_2$. By the end of the experiment, however, their abundance has dropped significantly, and other community members have outgrown these microbes.

Lastly, we conducted an additional, very similar experiment with just subject strain STR26155. In this final experiment we tested varying inoculation levels of subject strain STR26155 in an attempt to mimic the conditions of a very large accidental release contained in a small reservoir. A pre-culture was gently

pelleted by centrifugation and supernatant decanted to minimize nutrient carry-over into experimental flasks. The pellet was resuspended in sample water from two stations that had been passed through a 106µm sieve as before. This was then used to inoculate 1.25 L cultures to an OD of the subject organism of 0.05, 0.005, and 0.001. As before triplicate cultures were incubated in our La Jolla greenhouse and sampled weekly. As observed in the prior three experiments, for inoculation densities of 0.001 and 0.005 negligible differences in fixed carbon of the flasks were observed between treatments and control (**Figure F9**). At our greatest inoculation density tested both water sources tested resulted in an elevated level of TOC. This level is nearly equivalent however to the magnitude of additional TOC observed at $T_0$. We hypothesize that this result is largely due to the significant addition of macronutrients present in the biomass added at $T_0$. Microbiome profiling similarly indicates that no major shift in the microbiota has occurred at any of the inoculation densities tested. As observed in prior experiments, the microbial consortia most strongly cluster by water source, then by timepoint in the experiment and only somewhat by treatment level (**Figure F10**). Microbiome profiling allows us to track the sequence variant specific to subject *Parachlorella* STR26155. Utilizing reads counts as a semi-quantitative proxy for relative abundance, at $T_0$ for the highest inoculum tested the subject strain was eight and fifteen times more abundant (in IVF008 and IVF016, respectively) than the most abundant organism in the untreated water sample (which was a cyanobacterium). While still numerically dominant after one week, by two weeks *Parachlorella* STR26155 was no longer dominant and at only half and a tenth as abundant as the dominant microbe (respectively). This pattern held for the last week of the experiment.

In summary, we have conducted multiple “invasion”-type experiments with both the recipient and subject strain. These experiments employ local water samples collected over a span of nearly half a year to encompass some of the expected differences in conditions and microbiota over the course of an annual cycle. These experiments employ inoculation densities orders of magnitude higher than a catastrophic release could be envisioned to release. We do observe that SGI *Parachlorella* strains can persist in the face of competition from indigenous species. In no case, however, have we seen SGI *Parachlorella* strain dominate, alter the productivity of the system, or impact the microbial community structure.

# G. Information Applicable to Small-Scale Field Tests

## 1) Objective of the Tests

There is great promise in the potential to utilize photoautotrophic algae to produce a liquid fuel with a low-carbon footprint. For many years now, SGI has been utilizing the best wild-type strains we could find. Additionally, we have employed various classical improvement strategies to further improve our strains. Unfortunately, even our best wild-type and classically improved strains do not meet productivity metrics required to make them competitive with fossil fuels. As such, we also incorporate modern genetic engineering methods to produce engineered algae with beneficial traits aimed at the long-term goal of producing a low-carbon fuel.

Clearly, we need to understand if there is risk to humans and the environment as we work with engineered algae at greater and greater scales, and importantly in bioreactors open to the environment. Algae have been grown in open mass-culture since ancient times[3] with no clear negative environmental impacts. Notwithstanding, SGI understands the need to assess algae created using modern modification techniques for any new or unique risk potential.

There is little publicly available data on the potential dispersal of algae grown in open photobioreactors other than the very limited microalgae study described in the TERA submitted in 2013 by Sapphire Energy and the University of California, San Diego,[71] which examined the extent of dispersal of the host organism grown in open ponds during fifty days of outdoor cultivation. They observed that while the engineered algae dispersed from the cultivation ponds, colonization of the trap ponds by the GE strain declined rapidly with increasing distance from the source cultivation ponds. When inoculated in water from five local lakes, the engineered algae's effect on biodiversity, species composition, and biomass of native algae was indiscernible from those of the wild-type algae, and neither the engineered nor wild-type algae were able to outcompete native strains.[12]

Two additional microalgae-based TERAs have been approved by the U.S. EPA.[76-77] They were submitted by the Arizona State University, Arizona Center for Algae Technology and Innovation (AzCATI) in 2017/18, but at the time of SGI's application, AzCATI field work has not yet begun.

As we make near-term plans to test various engineered algae, furthering our knowledge in this area is crucial. As such, for this TERA we have generated an engineered strain with minimal intergeneric DNA. This strain was developed to have minimal discernable phenotypic differences relative to the recipient strain, except for possessing a nucleic acid signature and corresponding reporter protein to allow us to specifically track this strain in open-culture and in the environment. The main objective of the proposed test is to work with an engineered alga in open photobioreactors at a scale larger than prior work which begins to approach the expected scale needed for future commercial viability. We proposed to grow the subject strain in open "raceway" ponds" (described in-detail below) of 0.1-acre surface area in a manner that reasonably mimics what future production processes may be. This will enable the collection of real-world data on the potential for our algae to disperse, establish, and impact the local environment. This data is crucial to inform future applications wherein engineered algae with improved productivity phenotypes will be tested at increasing scale.

## 2) Nature of the Site

### I. Location and size of the test area

Experiments will be conducted at the CAAF. An aerial image of the facility with pertinent structures highlighted is provided in **Figure G1**. The CAAF is located on private land approximately three miles east of the Salton Sea in the unincorporated area of the County of Imperial, California. The physical address is 250 West Schrimpf Road, Calipatria, CA, 92233. The legal land description is: the northwest and southwest quarters of Section 19, Range 14E, Township 11S.

The facility's approximate geographic coordinates are N 33.198491 W 115.558857. It is bound on the north by McDonald Road and the Imperial Irrigation District's (IID) "O" Lateral and on the south by Schrimpf Road and the IID's "O" Drain. The "O" Lateral is fed by the All American Canal.

Regional access is provided from State Route 111, via McDonald Road. An existing driveway entrance is located on Schrimpf Road. A six-foot chain link fence surrounds the property, with a controlled-access gate on Schrimpf. An east-west six-foot chain link fence divides the property into two forty-acre sections. The northern section is not currently active. The site is staffed with 15-20 full and part time employees.

The specific area within the CAAF that will be utilized to operate the two 100,000-liter (0.1 acre) ponds is approximately one-half acre in size, located on the southwest part of the facility.

### II. Elevation and slope

The site rests at an elevation of 220 feet below mean sea level, on a plot of land that is exceptionally flat, sloping very gently downward to the west.[78] For reference, the surface of the Salton Sea is approximately 227 feet below mean sea level. A drainage study was commissioned by SGI in 2014.[79] Details are provided in **Section H.4.VI.**

### III. Proximity to water bodies

The site is located near (~three miles) to the Southeast corner of the Salton Sea (**Figure F2**). The nearest fresh water source (at a distance of ~1.5 miles) is the Alamo River, located to the Southwest of our facility. The site uses production water provided by the Imperial Irrigation District[q] (IID), which sources their water from the Colorado River. The IID transports river water from Yuma AZ, utilizing various open channel irrigation canals that network throughout the Imperial Valley. The site is designated as a zero-discharge facility meaning that none of the water taken onto the site is released back into in the local water system (with the exception of rainwater not falling into a pond or collection basin).

### IV. Prevailing winds

The prevailing winds at the site arise from the southeast. Summarized daily averages for one calendar year shows the strong frequency of winds from this direction **(Figure G2**). There is a less frequent, but moderately more intense wind pattern with winds coming from due west.

[q] https://www.iid.com/water

This variation in the prevailing wind is most prevalent in spring-time months, although not exclusively so. Hourly averages for the months of May through August (the anticipated months of the experimental release application) show a similar pattern to the yearly plot of daily values. Winds are predominantly from the southeast and generally more moderate during these months of the year.

## 3) Field Test Design

Seed stocks will be maintained in a dedicated grow room and transferred only between sealed containers during the scaling process. Once at least 100 L of seed has grown to a density of at least 1.0 g/L, the seed stock will be utilized to inoculate the 2,000 L and 4,000 L PBRs (**Figure G1**) at a density of approximately 0.1 g/L. Once the PBRs reach a density of at least 1.0 g/L, they will inoculate one of the 0.1-acre ponds at a target operational starting density of 0.1 g/L. These ponds will then run for one week each. At the end of a week of growth, the ponds will be deactivated and disposed as described in **Section G.4**.

Details of monitoring endpoints, procedures, and timelines are provided in detail in **Section J**. Briefly, while running the 0.1-acre raceway ponds in a production-like mode (although still for research and development purposes, and at a significantly smaller scale than full-scale biofuel production ponds) we will regularly sample multiple sample types from a variety of sites (e.g. bioaerosols, trap ponds, CAAF production ponds, local environmental sampling) to provide data on the potential release of the engineered alga from the experimental ponds. We will conduct active monitoring for one week prior to the start of open engineered alga cultivation, during the entire course of the experiment, and for 2 weeks following termination of the engineered alga ponds. During this active monitoring period, one type of endpoint will be the five 350 L “algae-trap” ponds established to help assess the dispersion capability of the subject organism. Additionally, we will sample regularly from all other ponds on site that are in active use and assay for the presence and abundance of the subject strain. Lastly, regular bio-aerosol samples will be collected and similarly assayed for the presence and abundance of the subject strain. Both during the active monitoring, and for one year following first inoculation, we will continue to carry out passive monitoring consisting of monthly sampling from our established environmental stations (detailed in **Section F**).

Samples will be collected daily for the CAAF Lab to perform growth measurements as described in **Table G1**. Briefly, these measurements will include optical density ($OD_{730}$), ash-free dry weight (AFDW), photosynthetic efficiency (PAM), total organic carbon (TOC), fatty acid methyl ester composition (FAME), microscopic analysis and metagenomic analyses. Excess samples will be disposed of in 0.5% sodium hypochlorite. The culture will be inoculated with media containing nitrogen, phosphorus, and trace minerals.

## 4) Methods of Cultivation

### I. Growth strategy

See **Section G.3**.

### II. Safety procedures and precautionary actions

As previously described, all staff will be trained in the cultivation of algae and will follow established SOPs, designed by SGI scientists and approved by the Institutional Biosafety

Committee, for the containment, labeling, and disposal of the engineered algae. Strict labeling and recording procedures will be followed over the duration of the experiment. The subject microorganism will be appropriately labeled with an orange label containing the strain nomenclature, and with a Laboratory Information Management System (LIMS) label containing strain ID, time of sampling, pond from which the sample was taken. Samples will be transported in leak-proof vessels with secondary containment.

III. Large culture deactivation

At the end of each experiment, the ponds will be deactivated-in-place with at least 4 mL/L of a 12.5% sodium hypochlorite solution before disposal in the site's evaporation pond. The effectiveness of the inactivation protocol is discussed in **Section I.IV**.

IV. Waste handling

The CAAF produces both hazardous laboratory waste and non-hazardous waste. There is no waste treatment on-site.

*Hazardous waste*

All laboratory biological waste is considered hazardous waste and will be disposed of into biological waste containers, then removed from the site and properly managed by a licensed hazardous waste vendor. The site holds both Federal and CAL/EPA registrations.

Hazardous materials on the site, including hazardous waste, are handled according to applicable state and federal laws, as administered by California and Imperial County Department of Environmental Health (DEH). The types, quantities and use / storage locations of hazardous materials are identified in the site's Hazardous Material Business Plan (HMBP), as reviewed and approved by the Imperial County's Certified Unified Protection Agency (CUPA), California Department of Toxic Substances Control (DTSC), and county Fire Marshal. Separate annual inspections are performed by both the Imperial County DEH and the Fire Marshal.

Current operations do not generate chemical vapors and operations under the TERA are not expected to generate chemical vapors. Small quantities of volatile solvents and petroleum-based fuels (<55 gallons) are managed under the HMBP and do not require an air permit. An air permit has been issued by the Imperial County Air Pollution Control District for the diesel engine running the fire protection system pump.

*Non-hazardous waste*

Solid non-hazardous process waste, from either direct collection or via solids from the evaporation pond, is landfilled either in a Class-II or Class-III facility, depending upon the water content, according to applicable Federal and State laws. Chemical analyses provided quarterly to the State has demonstrated that the waste does not include hazardous substances, does not include F- or K- listed waste, but does contain constituents that have the potential to affect water quality, specifically, high sodium chloride (salt) content. The solids from the evaporation pond are referred to as a "designated waste" pursuant to California water law.

Evaporated salt waste material that is >50% water can be shipped via licensed hauler in lined dump trucks to a licensed Class-II landfill for disposal (lined to contain liquids). However, the preferred means of disposal will be to allow the material to dry below 50% water, and when the dried material passes the EPA "paint filter test" it will be shipped via a licensed vender in unlined trucks to a licensed Class-III landfill. A Special Waste Profile has been approved by a local landfill.

*Evaporation pond*

All process liquid waste is piped to an evaporation pond with a total capacity of 8.6 acre-feet (AF). The pond is permitted by the California Water Quality Control Board Region #7.[80] The pond was designed to comply with Federal, State and County construction standards. Quarterly Reports on the evaporation pond physical integrity, chemical composition and water levels are provided to the State.

The pond measures approximately 260' X 260' at the top of pond running east-west, covering an area of approximately 1.5 acres. From the "Top of Pond", elevation drops quickly over the bank a total of six feet at the northeast corner. The bottom of the pond slopes downward at 1% grade towards the southwest corner, which is the deepest point at eight feet. The bottom of pond measures approximately 200' X 200' and covers an area just under one acre. Located at the southwest portion of the South Pond is a leak collection and recovery system (LCRS) sump. Total pond capacity (to 99") is approximately 36,4474 cubic feet (CF), and volume to the 24" freeboard elevation is 245,809 CF. The pond was designed to accommodate the effluent from algae cultivation as well as 100-year flood event[79] (defined as 3" rainfall within a 24-hour time period).

The evaporation pond system was designed and constructed to comply with California Code of Regulations (C.C.R.) Title 27 Environmental Protection, Division 2 Solid Waste.[r] The construction standard was for a Class II Surface Impoundment. The heavy-duty dual-liner system contains a leak detection system and biogas-conduction grid system.

*Evaporation pond liner detail*

The overall liner configuration (**Figure G3**). Over existing low-permeability soil, the liner is built in four-layers:

1) Top Layer: Raven-reinforced[s] 45-mil thickness linear low density polyethylene (LLDPE) membrane liner;
2) Second Layer: Double-sided geocomposite material consisting of a geogrid bonded top and bottom to two layers of six ounces / square yard nominal weight geotextile fabric. This provides abrasion resistance to the top layer and allows for leak detection;

[r] https://www.calrecycle.ca.gov/laws/regulations/title27

[s] https://ravenefd.com/

3) Third Layer: Raven reinforced 45-mil thickness LLDPE membrane liner; and

4) Bottom Layer: Double-sided geocomposite material consisting of a geogrid bonded top and bottom to two layers of six ounces / square yard nominal weight geotextile fabric. This provides additional abrasion resistance and allows for gas venting which prevents liner "whaling". Carbon dioxide gas is well known to emanate from the soil in this geologically-active area.

V. Worker safety procedures

There will be three to four workers involved in the initial application and three to four workers involved in the subsequent activities (e.g. sampling, pond monitoring.) All the activities and duration of these activities are described in **Table G2**.

Routine pond monitoring will be accomplished by three to four people at the CAAF site and will include taking a set of samples daily (approximately one hour/day activity time). Daily sample processing at the field site will take three to four people approximately two hours each day and includes microscope observations, $OD_{730}$, AFDW, TOC, FAME, and metagenomic analyses.

Routes of exposure for all activities include skin contact and eye/nose/mouth from possible splashes, which will be limited using proper personal protective equipment (PPE), as required by SGI regulations. Proper PPE includes: gloves, safety glasses, long pants, and steel-toed shoes.

## 5) Monitoring Endpoints and Procedures for Isolating/Detecting the TSCA Subject Microbe

See **Section J** below.

## 6) Sampling Procedures

Approximately 100 mL of sample will be taken from the pond every day. This sample will be utilized to perform several analyses as shown in **Table G1**. Excess sample will be bleached before disposal.

Each sample will be labeled as previously described in **Section G.4**. Briefly, each sample will have a Fluorescent green label and an associated LIMS label.

I. Methods of measurement and equipment used

Biomass density is assessed in three ways. Optical density at 730nm ($OD_{730}$) is an established analogue for cell density. $OD_{730}$ is performed on a Molecular Devices SpectraMax 384 Plus plate reader in a 96 well microtiter plate using seawater media as a reference. Measurements are performed on raw culture samples as well as 10x dilutions as it has been observed in the SGI labs that 10x dilutions are more accurate at densities above 0.4 due to the technical limitations of the instrument. Accuracy of the SpectraMax 384 Plus is determined via validation of standard samples that are sent from the SGI Greenhouse in La Jolla.

The second method utilized to quantify biomass is Ash Free Dry Weight (AFDW). This is a modified total suspended solids methodology in which samples collected from the pond are filtered through a Whatman GF/C glass microfiber filter and rinsed with 2 M ammonium formate to remove any extracellular salt or particulates. The samples are then dried in a 105 °C

oven, weighed, and baked at 550 °C to volatilize any organic material before a final weight of the filter is recorded. This allows for a quantification of total biomass as well as ash in the ponds. Accuracy of the AFDW methodology is determined via validation of standard samples that are sent from the SGI Greenhouse in La Jolla.

The final method utilized to quantify biomass is total organic carbon (TOC). Samples collected from the pond are diluted in reverse osmosis-purified water and injected onto a TOC analyzer. Total carbon in the sample is pyrolyzed to $CO_2$, which is measured by IR spectroscopy. Inorganic carbon is determined via acidification of a portion of the sample to convert carbonates to $CO_2$, which is then purged from the acidified sample and measured. TOC is calculated by the subtraction of IC from TC. Each TOC instrument run is accompanied by quality control standards that are listed in the TOC SOP to ensure accuracy of values across runs.

Photosynthetic yield is measured with a Walz MINI-PAM Photosynthesis Yield Analyzer. This instrument provides an assessment of the effective quantum yield of photochemical energy conversion in photosynthesis.

### II. Methods for the statistical analysis of field data

Data collected from the experiment will be utilized to calculate biomass accumulation rates on a total biomass basis (AFDW) and Total Organic Carbon Basis (TOCs). These rates will be compared to weather data as well as other abiotic conditions to determine effects of abiotic conditions on growth rates at scale. Growth rates in the 0.1-acre ponds will be utilized to compare versus previous data sets to determine correlation between various scales, and to determine applicability of strain for production at large scale.

## 7) Measurement Methodologies and Quality Assurance/Quality Control

Data collected during the experiment is entered by both scientists and technicians into lab notebooks, forms and the LIMS system, depending upon the data type, and monitored for quality by dedicated research assistants, before final submission of the data in accordance with 40 C.F.R. § 725.250(f)(2). With a scientist's oversight, research assistants evaluate the quality of data and initiate procedures for the repetition of analyses on an as-needed basis.

## 8) On-Site Containment Practices

All on-site workers will be trained to follow the SOPs described below. The CAAF facility has standard training procedures for workers in all roles, and role-specific training will be provided and documented prior to a worker beginning that specific role, and then on a periodic basis as a refresher or if the SOPs have been revised.

### I. Transportation of cultures

The subject microorganism was created within the labs at SGI. The strain is then transported to the SGI La Jolla Greenhouse (within the same research park) in sealed secondary containers. There, the cultures are maintained and scaled prior to movement to the CAAF. Shipment of the subject microorganism will be made in clearly-labelled, sealed containers of approximately one to three liters. These will be further contained in secondary spill-proof containers and transported with enough bleach to neutralize the cultures in the case of a catastrophic failure.

The transportation kit will also include nitrile gloves and materials to assist in the cleaning of any spills during transportation. The cultures will only be removed from containers once they reach the inside of the grow room at the CAAF facility. All vessels containing the subject microorganism will be labeled with Fluorescent Green stickers for rapid identification of the SGI strain designation, and to indicate that the vessel contains an engineered microorganism. All cultures are logged prior to departure from the La Jolla Greenhouse and upon arrival at the CAAF.

All shipments to and from the CAAF of the subject microorganism include (1) shipping manifest, (2) aquaculture registration authorizing intra-state transport, (3) Safety Data Sheet, (4) shipping SOP, and (5) the spill management SOP with emergency contact list.

II. Cleaning and disinfection

To ensure that the subject microorganism is completely removed from the test site after the experiment has been completed, all liquid biomass will be treated with 4 mL/L of 12.5% sodium hypochlorite for at least one hour prior to disposal. This dose is 12.5-fold greater than the experimentally determined effective dose for killing both recipient and subject strains. Scale up vessels, including Fernbach flasks and carboys, will be treated with bleach to neutralize the microorganism before dumping down the drain to the evaporative pond. Carboys will be cleaned and autoclaved for reuse. 0.1-acre ponds will be deactivated in place with bleach before disposal into the evaporative pond. Samples that have been collected from the site will be neutralized by treatment with 4 mL/L of 12.5% sodium hypochlorite for a minimum of one hour before disposal. The effectiveness of this inactivation protocol is discussed in **Section I.2.IV**.

III. Containment features

Seed stocks will be maintained in the CAAF laboratory in a dedicated culture area that is designed to capture all drips and spills. Cultures will only be transferred in closed containers during the scale up process to minimize culture exposure. Enclosed carboys will be utilized to inoculate 2,000 L and 4,000 L PBRs adjacent to the 0.1-acre ponds. PBRs and ponds have secondary containment in the form of a 24-inch berm that is lined with a mesh reinforced, puncture resistant, UV-resistant material. The berm has an effective footprint of 1 acre and can hold the approximately 5x the capacity of the two 0.1-acre L ponds plus all PBRs, in the highly unlikely scenario of complete primary containment failure.

IV. Site access

The CAAF facility is surrounded by a fence that is always locked and access is limited to SGI staff. All on-site workers will be trained to follow established SOPs. These SOPs and trainings are required by SGI for all workers at the CAAF location and cover general lab safety as well as cultivation, transportation, and disposal practices for the subject microorganism. Visitors, guests, and vendors are all required to sign in and are accompanied at all time by an SGI employee to ensure safety and limit access to areas of the site containing the subject microorganism.

V. Response to adverse conditions

Weather for the CAAF is constantly monitored by an on-site weather station and through news media weather forecasts. In the event where highly adverse weather conditions are likely to arise, such as heavy rains or high winds, the site management will make a determination as to whether the inactivation of any ponds or PBRs, or mitigation by other methods, are necessary to minimize the potential loss of primary containment.

Staff is trained to follow an emergency response SOP and to respond to any failures by dosing the area with at least 4 mL/L of 12.5% sodium hypochlorite, before transferring liquids to the evaporation pond. The secondary containment around the 0.1-acre ponds, 2,000 L and 4,000 L PBRs is designed to hold 5x as much fluid as will be present in the ponds. Barrels of bleach will be stored on a dedicated pad with a pump adjacent to the secondary containment area that is sufficient to neutralize all of the biomass within the ponds and PBRs.

VI. Biological containment

There will be no biological containment features included in the experimental design of the field trial as there is no known method to 'trap' algae species. We have not incorporated any biological containment features into the subject organisms through genetic engineering.

In addition to the standard GLSP containment criteria, SGI will also install, in an abundance of caution, bird netting over the 0.1-acre ponds to prevent distribution of the algae by other fauna, such as birds. However, for larger ponds, bird netting will not likely be feasible and alternative measures and/or deterrents will be evaluated.

VII. Frequency and types of on-site observations

**Table G1** provides details on the data collection for ambient or adverse weather effects to be recorded by the onsite automated weather station.

## 9) Termination and Mitigation Procedures

I. Termination procedure

Once the field experiment has been terminated, all biomass will be inactivated by bleaching the cultures with at least 4 mL/L of 12.5% sodium hypochlorite for at least one hour prior to disposal. All equipment will be cleared of the microorganism (including sample containers, ponds, PBRs, etc.) by bleaching or autoclaving and will be discarded as necessary. Any pond spills will be contained within the secondary containment and treated with bleach. The liquid will then by disposed of into the evaporative pond at the CAAF site.

II. Identifying conditions for emergency termination

As detailed briefly above and in greater detail in **Section J** below we will conduct bi-weekly sampling for monitoring of our subject organism. This includes sampling from the five established algae trap ponds that will be used to assess the dispersion capability of the subject microorganism. Additionally, all ponds in use at our facility will also be sampled bi-weekly to monitor for the presence of the subject alga. If the subject strain is detected in any algae trap or production ponds on-site, follow-on sampling will continue. If we detect a 1,000-fold increase of the subject microorganism (by qPCR) above its originally detected titer, this would indicate

that the subject strain has established itself and is actively proliferating. In this case we shall consult EPA for further action and be prepared to terminate the experiment if necessary.

III. Emergency termination procedures

Four 55-gallon barrels of concentrated bleach (12.5% sodium hypochlorite), containing sufficient material to inactivate double the production capacity of all the ponds and PBRs located within secondary containment area, will be stored immediately adjacent to the containment area. In the event of adverse environmental events or unanticipated emergencies, the experiment will be rapidly terminated by pumping the bleach into the appropriate pond or PBR, using the applicable SOP.

IV. Describe how spills or leaks will be handled

All spills will be treated with bleach either transferred to the evaporation pond or handled with the site's solid waste disposal procedure.

# H. Manufacturing Process Descriptions and Production Volumes

## 1) Heterotrophic Fermentation

Not applicable. The *Parachlorella* species used in this study uses sunlight as its energy source, hence it is classified as a phototroph and not a heterotroph, which require carbohydrate sources for energy.

## 2) Photobioreactors (PBRs)

### I. Number/volume of PBRs

Six PBRs will be in use over the duration of the experiment. Three PBRs will contain 2,000 L and three will contain 4,000L.

### II. PBR design and arrangement of PBRs at the site

PBRs are of a patented SGI design. Briefly, the PBRs are of a horizontal design and run for 200 feet (2,000 L) or 400 feet (4,000 L) in the east to west direction.

### III. Size/volume/cell density – and whether batch or continuous culture

PBRs are approximately 200 feet (2,000 L) or 400 feet (4,000 L). Cultures are grown in batch conditions from a density of 0.1 to 1 g/L.

### IV. Number of harvests per year and time between harvests (batches)

PBR batches will be inoculated every two weeks, grown for two weeks, and then utilized as seed to start the 0.1-acre ponds. No harvests will be conducted over the duration of this experiment.

### V. Number of microorganisms harvested – production alga and contaminants/pathogens

No harvests will be conducted over the duration of this experiment.

### VI. Harvesting technologies

No harvests will be conducted over the duration of this experiment.

### VII. PBR material (thickness, mil, tensile strength, etc.)

The PBRs are made of 10 mm reinforced, food-grade polypropylene/nylon-blend tubing.

### VIII. Integrity/weatherability of materials used in PBRs

Plastic tubing used for the PBRs are tear resistant and have an operational lifespan of 180 – 240 days.

### IX. Longevity/replacement time of PBRs

Plastic tubing used for the PBRs have an operational lifespan of 180 – 240 days before replacement.

### X. Junctions of inlet and outlet tubing and potential for leaks

The inlet and outlet of the unit are outfitted with PVC boots in which the plastic PBR tubing is stretched over and sealed. Within the boot are inlet and outlet hoses as well as sealed probe sockets. PBRs are checked daily for leaks and sealed with silicone epoxy if visual tears are

observed in the plastic. PBRs are housed on top of half cylinders of PVC that catch any spills or leaks that occur along the length of the PBR.

XI. Biofuel or bioproduct produced

No biofuels or bioproducts will be produced over the course of this experiment.

XII. Amount and source of $CO_2$ and potential contaminants (e.g., metals in flue gases)

PBRs are maintained at a pH of 7.0 using a $CO_2$ based control system. Commercial grade $CO_2$ is utilized and stored onsite in a 32-ton liquid $CO_2$ tank.

XIII. Amount and sources of supplied nutrients

Cultures are supplied with modified F/2 Media from the commercially available Proline A&B Media Components from Pentair (Apopka, FL). Media consists of 1.3 mL/L of each component. Salt is added to the PBRs at a concentration of 35 g/L.

XIV. Water source

Water is sourced from the Colorado River and processed on site through 0.1 µm sand filters followed by ozone treatment before use in the PBRs.

XV. Water characteristics

We have monitored the water quality at CAAF by regular sampling of the IID canal which feeds our facility. We have contracted a third-party laboratory for these analyses, who apply EPA 200.7 for elemental analyses, or other ELAP-approved methodologies. Summarized in **Table H1** are data from eight monthly sampling events spanning March 2018 through November 2018.

XVI. Characteristics of algogenic organic material (AOM)

Proximate biomass composition of *Parachlorella* STR00012 and lipid profiles are provided in **Figure A4.**

XVII. Distance to surface and underground water sources

Details of nearby groundwater and surface waters are provided in detail at the end of this section in “Additional Siting Info.”

XVIII. Use of antimicrobials or pesticides in media

The growth media utilized contains no antimicrobials or pesticides.

XIX. Inactivation methods

Strains are inactivated using 4 mL/L of 12.5% sodium hypochlorite for a contact time of no less than one hour.

XX. Releases of wastewater

The CAAF is a zero-discharge site. Post inactivation, PBRs are pumped to an onsite evaporation pond.

XXI. Disposal of spent biomass/use of spent biomass

At the end of the experiment, biomass will be inactivated using 4 mL/L of 4.0% for a contact time of no less than one hour. PBRs will then be pumped to an onsite evaporation pond.

XXII. Cleaning of PBRs for re-use or disposal of PBRs

After PBRs are decommissioned and spent biomass has been removed, the 10 mL plastic tubing is discarded as waste. PVC boots, tubing, and probes are decontaminated with 4.0% sodium hypochlorite solution and are re-used with new plastic tubing.

XXIII. By-products

No known by-products will be generated over the course of this experiment.

3) Open/Raceway Pond Construction and Design

I. Number/volume of ponds

Two ponds of 100,000 L will be used for this experiment.

II. Pond size/dimensions/surface area

Each of the two 0.1-acre ponds has a surface area of 0.1 acre.

III. Size/volume/cell density – and whether batch or continuous culture

The 0.1-acre ponds will be operated in batch conditions with culture growing from a density of 0.1 g/L to 1 g/L.

IV. Number of harvests per year and time between harvests (batches)

Batches will be produced every week, alternating between production ponds. There will be no harvests over the duration of the experiment.

V. Amount of microorganisms harvested – production alga and contaminants/pathogens

There will be no harvests over the duration of the experiment.

VI. Harvesting technologies

There will be no harvests over the duration of the experiment.

VII. Pond construction materials

The pond will be constructed from LLDPE, scrim reinforced and bonded liner. Areas around the paddlewheel will be constructed out of reinforced concrete and sealed with an epoxy sealant.

VIII. Use of liners

The 0.1-acre ponds are housed within secondary containment lined with mesh reinforced, UV resistant, puncture resistant material.

IX. Use of berms

The 0.1-acre ponds are enclosed within a two-foot berm that has an effective footprint of one acre.

X. Circulation system and rate and potential for bioaerosols

Standard paddlewheels are used in raceway ponds to provide circulation. Though overall minimal, the formation of aerosols is most likely to occur from the paddlewheels as they move in and out of the water column and through aeration/carbonation of the ponds.

We have begun to develop methods for measuring the presence and abundance of production algae strains in aerosol samples. During the course of this TERA experiment we anticipate that we will generate the data that will help us to better understand this.

XI. Biofuel or bioproduct produced

No biofuels or bioproducts will be produced over the course of this experiment.

XII. Amount and source of $CO_2$ and potential contaminants

Ponds are maintained at a pH of 7.0 using a $CO_2$-based control system. Commercial grade $CO_2$ is utilized and stored onsite in a 32-ton liquid $CO_2$ tank.

XIII. Amount and sources of supplied nutrients

Cultures are supplied with modified F/2 Media from the commercially available Proline A&B Media Components from Pentair (Apopka, FL). Media consists of 1.3 mL/L of each component. Salt is added to the ponds at a concentration of 35 g/L.

XIV. Water source (e.g. fresh water, salt water, wastewater, recycled water)

Water is delivered from the Imperial Irrigation District canal system sourced from the Colorado River, and processed on site through 0.1 µm Sand Filters followed by ozone treatment before use in the ponds.

XV. Water characteristics

We have monitored the water quality at CAAF by regular sampling of the IID canal which feeds our facility. We have contracted a third-party laboratory to perform analyses under EPA methods (200.7 for elemental analyses) or other ELAP-approved methodologies. Summarized in **Table H1** are data from eight monthly sampling events spanning March 2018 through November 2018.

XVI. Characteristics of algogenic organic material (AOM)

Proximate biomass composition of recipient *Parachlorella* strain STR00012 and lipid profiles are provided in **Figure A4.**

XVII. Distance to surface and underground water sources

Details of nearby groundwater and surface waters are provided in detail at the end of this section in “Additional Siting Info”.

XVIII. Use of antimicrobials or pesticides in media

No antimicrobials or pesticides are utilized in the growth media.

XIX. Inactivation methods

At the conclusion of the experiment, the cultures will be inactivated with 4 mL/L of 12.5% sodium hypochlorite for at least 1 hour.

XX. Releases of wastewater

The CAAF is a zero-discharge site. Post inactivation, PBRs are pumped to an onsite evaporation pond.

XXI. Disposal of spent biomass

At the end of the experiment, biomass will be inactivated using 4 mL/L of 12.5% for a contact time of no less than one hour. Ponds will then be pumped to an onsite evaporation pond.

XXII. Disinfection of ponds between batches

At the conclusion of the experiment, after all the inactivated biomass has been removed, any areas where visible biomass has adhered to the sides of the pond will be sprayed with 4.0% sodium hypochlorite and scrubbed from the side of the liner with brushes. After cleaning has concluded, brushes will be decontaminated with 4.0% sodium hypochlorite.

XXIII. By-products

No known by-products will be generated over the course of this experiment.

## 4) Additional Siting Information for Commercial-Scale PBRs and Open Ponds

### I. Location

Experiments will be conducted at SGI's CAAF. An aerial image of the facility with pertinent structures highlighted is provided in **Figure G1**. The CAAF is located on private land approximately three miles east of the Salton Sea in the unincorporated area of the County of Imperial, California. The physical address is 250 West Schrimpf Road, Calipatria, CA, 92233. The facility's approximate geographic coordinates are N 33.198491 W 115.558857. The lands surrounding SGI's facility are semi-rural, containing wetlands, farmland, and industrial operations (geothermal and other renewable energy). Outdoor recreational activities include fishing, hunting and boating. A few inhabited dwellings are within a 3.5-mile radius of the site.

The CAAF is situated in an ideal location for algal production in an area with favorable temperatures and high solar irradiance. It was also chosen due to its proximity to the Synthetic Genomics' main offices located nearby in San Diego, California. The site has been primarily used as a commercial algae cultivation facility since the late 1970s. SGI purchased this approximately seventy-five-acre property from the Carbon Capture Corporation in April 2012. Various species of algae such as *Dunaliella salina* (for β-carotene production), *Arthrospira platensis* (aka *Spirulina*, for protein and phycocyanin production), and *Haematococcus pluvialis* (for astaxanthin production) have been grown at the site. The site has also taken part in several research and develop programs funded by the Department of Defense (DOD) and the Defense Advanced Research Project Agency (DARPA) for the creation of biofuels. This facility has previously been certified by the National Sanitary Foundation for compliance with Current

Good Manufacturing Practices (GMPs) for SGI's production of nutritional supplements and other food ingredients from microalgae.

II. Land Use

The County of Imperial General Plan designates the site as Agriculture, and it is zoned A2R-G (General Agriculture, Rural, with Geothermal Overlay). The SGI facility operation is deemed to be consistent with the Land Use Element of the county's General Plan,[81] and also deemed consistent with the county's Land Use Ordinance[82] which states: "Aquaculture for the growing and harvesting of algae, fish, frogs, shrimp and similar aquatic products is a use that is permitted 'by right.' This includes shipping but does not include processing" [emphasis added]. The land immediately west of the property is zoned medium industrial (M2G-PE).

The California Department of Fish and Wildlife issued an aquaculture registration to the CAAF in 2012 which is renewed annually (**Figure F1**). The registration addresses environmental risks, facility design and operation, algal strain movement, open cultivation of specifically-approved algal genera, and waste handling.

III. Local population centers

The City of Calipatria is approximately six miles south. Calipatria' s population is approximately 7,700 people. This includes approximately 4,000 inmates at Calipatria State Prison, which is approximately five miles southeast of our facility.

The City of Niland (population ~1,000) is approximately 3.5 miles northeast.

There is one private residence, a small farm two miles east on the southwest corner of Rt. 111 and Schrimpf Road, which is situated closer to our facility than the other small population centers of Niland and Calipatria.

IV. Local industrial and other sites

GeoGenCo LLC owns the property bordering SGI immediately to the east. A ten-acre cut-out of SGI's property holds three geothermal wellheads which will feed a permitted small 15 MW closed-loop geothermal power plant sited on land directly bordering the CAAF to the east. The CAAF and surrounding lands have generally poor-quality saline-sodic soil (high pH and heavy with salts) not suited for commercial agriculture. The GeoGenCo property is usually fallowed, but a portion of it has been used for production of hay since the fall of 2017. Further east and northeast are approximately 500 acres of "managed marshlands", maintained by the Imperial Irrigation District.[t]

Bordering the SGI directly west is a fallowed 150-acre parcel, formerly used for duck hunting. Its dried ponds running north-south are visible via satellite imagery. Further west of the fallowed parcel (1.1 miles from SGI) is the 50 MW Hudson Ranch I (aka Featherstone) geothermal power plant[u]. West of the geothermal plant is the Salton Sea, approximately 3.5 miles west of SGI[v],

[t] https://www.iid.com/water/library/qsa-water-transfer/mitigation-implementation/managed-marsh-qsa-related-documents

[u] https://www.energysource.us.com/

[v] http://saltonseaauthority.org/get-informed/facts/

and the Sonny Bono Salton Sea National Wildlife Refuge,[w] which is approximately 3.5 miles southwest of SGI.

There are active private duck hunting clubs immediately south and southwest of SGI's facility (across Schrimpf Rd.) A local algae products company, Earthrise Nutritionals, operates a Spirulina algae operation 3.5 miles to the southeast of SGI.

V. Climate, precipitation, winds, storms

The facility resides within the Colorado Desert ecoregion, an area with vegetation and habitat that has adapted to an arid sub-tropical climate. Elevations in this ecoregion range from 230 feet below sea level at the Salton Sea to 2,200 feet above sea level at the boundary with the Peninsular Ranges. Vegetation in the Colorado Desert ecoregion is supported by an average annual precipitation of approximately 5 ½ inches.

Imperial County is one of the hottest and driest parts of California, best described as a low latitude desert characterized by hot, dry summers and relatively mild winters. Average annual precipitation in Imperial County is less than three inches.[x] Daily average temperature in winter ranges between 65 °F and 75 °F. During winter months it is not uncommon to record maximum temperatures of up to 80 °F. Summers are extremely hot with daily average temperature ranges between 104 °F and 115 °F, with maximum temperatures up to 120 °F.

During the summer, due to the presence of the Pacific high-pressure zone off the coast of California, a thermal trough develops over California's southeast desert region. The intensity and orientation of the trough varies from day to day. Although the mountainous terrain surrounding the Imperial Valley inhibits air circulation, the influence of the trough does permit some inter-basin exchange of air with coastal locations through the mountain passes. Relative humidity in the summer is very low, averaging 30% to 50% in the early morning and 10% to 20% in the afternoon. During the hottest part of the day, a relative humidity level below 10% is common. However, the effect of extensive agricultural operations in the widely-irrigated Imperial Valley tends to increase local humidity. The prevailing weather conditions promote intense heating during the day in summer, with marked cooling at night.

The prevailing winds at the CAAF site come out of the southeast. Summarized daily averages for one calendar year shows the strong frequency of winds from this direction **(Figure G2**). There is a less frequent, but moderately more intense wind pattern with winds coming from due west. This variation in the prevailing wind is most prevalent in spring-time months, although not exclusively so. Hourly averages for the months of May – August (the anticipated months of the experimental release application) show a similar pattern to the yearly plot of daily values. Winds are predominantly from the Southeast and generally more moderate during these months of the year.

[w] https://www.fws.gov/refuge/sonny_bono_salton_sea/

[x] https://www.usclimatedata.com/climate/imperial/california/united-states/usca0508

## VI. Additional siting information

*Cultural resources*

The project site is located in the Salton Trough physiographic province and is underlain by geologic units composed of quaternary lake deposits of ancient Lake Cahuilla. These lakebed deposits have yielded paleontological fossils of freshwater shell beds, fish, seeds, pollen, diatoms, foraminifera, sponges, and wood from numerous localities in Imperial Valley. Vertebrate fossils such as birds, horses, bighorn sheep and reptiles also have been recovered from the area. The consequence of the area's rich paleontological resources is high paleontological sensitivity of the facility site.

These ancient lake sediments are thought to be entirely of Holocene age (approximately 12,000 years ago until the present) and, while not considered sensitive for significant fossil localities, may contain cultural resources because humans occupied North America for at least the past 14,000 years. Cultural resources studies have been conducted in the area neighboring the SGI facility. A part of this study included a search of records documenting previously recorded sites in the area. There are no previously recorded cultural resources within the SGI property boundaries.[83]

*Biological resources*

The Imperial Valley of California is a naturally arid region receiving only three inches of rain per year, on average. Natural desert habitats in the area support unique wildlife adapted to water scarcity and high temperatures. The agriculture development in the CAAF region provides abundant habitat for birds, small mammals, and amphibians. The Imperial Wildlife Area lies near the CAAF and the nearby Salton Sea, providing habitat for aquatic species unique to the area.[y] The Salton Sea and surrounding wetlands and uplands - particularly the Sonny Bono Salton Sea National Wildlife Reserve - also presents nesting, overwintering, and stopover habitat for many species of migratory birds in the Pacific Flyway; for some species, the seasonal Salton Sea flock is a large fraction of the species' global population.[84]

*Birds*

The County of Imperial is located on the Pacific Flyway for migratory waterfowl, shorebirds, and songbirds. Although this area is considered to be part of the Colorado Desert, approximately 500,000 acres of the Colorado Desert in the County of Imperial, including the facility site, have been converted to agricultural use.[z, aa] The irrigation system in the Imperial Valley attracts many bird species that are typically found in agricultural areas, including waterfowl, gulls, herons, cranes, ibises, egrets, doves, quail, sparrows, juncos, and finches. Some raptor species forage in this area as well, particularly the Western Burrowing Owl

[y] https://www.wildlife.ca.gov/Lands/Places-to-Visit/Imperial-WA

[z] https://www.nass.usda.gov/Publications/AgCensus/2012/Online_Resources/County_Profiles/California/cp06025.pdf

[aa] https://www.co.imperial.ca.us/AirPollution/Forms%20&%20Documents/AGRICULTURE/QuickFactsAboutIVag.pdf

(*Athene cunicularia hypugea*), which also uses burrows in many of the irrigation canals and drains.

Although the Western Burrowing Owl is not listed as a California or Federal Endangered Species, the Federal Government and the State of California Department of Fish and Wildlife list them as a species of special concern. They are also protected by the Migratory Bird Treaty Act of 1918. Importantly, the local community holds it with a special fondness and extra care is taken to avoid disturbing them.[bb]

Burrowing owls are frequently observed early in the day hunting rodents, reptiles and insects along the canals bordering Schrimpf Road. They are occasionally seen on the southwest corner of SGI's property. They have avoided SGI's operations, and SGI has not found evidence of burrows on its property. They have been documented on properties adjacent to the SGI facility, as well in the O-drain.[84]

*Fish*

There are fish within the SGI facility, in the freshwater inlet holding pond. The Imperial Valley irrigation waters are documented to host fish families including *Poeciliidae* (mollies) *Cyprinidae* (pupfish, carp, goldfish), *Centrarchidae* (bass and sunfish) and *Ictaluridae* (catfish) of which only the pupfish is native.[cc] Another native species, striped mullet, may reside in the Salton Sea.

There are reports of the Federally Endangered desert pupfish (*Cyprinodon macularius*) in and around the Salton Sea.[dd] The desert pupfish tolerates an extreme range of environmental conditions: salinities ranging from freshwater to 68-70 parts per thousand (ppt) for eggs and adults, and 90 ppt for larvae.; water temperatures as high as approximately 108 °F, with the lowest recorded temperature of approximately 40 °F; and oxygen levels down to 0.1 parts per million (ppm). Desert pupfish can also survive rapid changes in salinity and daily water temperature fluctuations of 72 °F to 80 °F and often escape stressors by diving into the substrate. There are no indications of any desert pupfish on the CAAF site.

A recent IID study of the Red Hill Bay Restoration Project documented the presence of the Desert Pupfish at the margins of the Salton Sea to the west and southwest of the SGI facility, including the western, downstream side (towards the Salton Sea) of the "N" lateral agricultural drain gate and canal.[85] This gate is located at the corner of Davis and Schrimpf roads across from the geothermal seep field (N 33.198316, W 115.579984).

[bb] http://www.ivcommunityfoundation.org/our-grants-&-programs/burrowing-owl-stewardship-and-education-fund/

[cc] http://calfish.ucdavis.edu/location/?ds=697&reportnumber=1294&catcol=4703&categorysearch=Imperial

[dd] https://www.wildlife.ca.gov/Regions/6/Desert-Fishes/Desert-Pupfish

During rare heavy rainfall events, runoff from SGI's facility will flow through an IID sump located at the property's southwest corner (N 33.198586, W 115.562499). This outfall is approximately one mile upstream of the "N" Drain gate where pupfish have been observed.

*Mammals*

Small mammals occupy habitat along the canals and drains. Some of the common species include western harvest mouse (*Reithrodontomys megalotis*), house mouse (*Mus musculus*), Norway rat (*Rattus norvegicus*), valley pocket gopher (*Thomomys bottae*), brush rabbit (*Sylvilagus bachmani*), striped skunk (*Mephitis mephitis*), raccoon (*Procyon lotor*), and muskrat (*Ondatra zibethicus*). Surrounding desert areas provide habitat for these species as well as larger mammalian species such as black-tailed jackrabbit (*Lepus californicus*), mule deer (*Odocoileus hemionus*), wild burro (*Equus asinus*), gray fox (*Urocyon cinereoargenteus*), coyote (*Canis latrans*), bobcat (*Lynx rufus*), and mountain lion (*Puma concolor*).

*Reptiles and amphibians*

Reptiles and amphibians typically associated with the Colorado Desert may occur in Imperial Valley agricultural areas. Some common species include Sonoran gopher snake (*Pituophis catenifer affinis*), western diamond-backed rattlesnake (*Crotalus atrox*), Marcy's checkered garter snake (*Thamnophis marcianus marcianus*), Great Plains toad (*Anaxyrus cognatus*) and the California desert tortoise (*Gopherus agassizii*).

*Threatened or endangered animals*

The Salton Sea area is habitat to several sensitive or endangered species:

*Federally endangered species*

Desert pupfish – the fish is endangered wherever occurring, including in the agricultural drain habitats present in the Imperial Valley; however, habitat conservation plans have been made only for Coachella Valley immediately to the north and for Pima County, AZ.

Yuma clapper rail – range includes the southeast portion of Salton Sea; no critical habitat rules have been established. The species is included in recovery and conservation plans for various water bodies including the Salton Sea.[ee]

*California bird species of special concern*

Burrowing owl is common in Imperial Valley (about 4,000 breeding pairs).[ff]

[ee] https://www.fws.gov/southwest/es/arizona/documents/recoveryplans/yumaclapperrail.pdf

[ff] http://ca.audubon.org/birds-0/burrowing-owl

Mountain plover is a migratory species that overwinters in Salton Sea.[gg]

The immediate environs of CAAF are not expected to host threatened or endangered species listed by the State of California or the U.S. Fish and Wildlife Service, but may be near or impact critical habitat for a number of species. Critical habitat units support important habitat and often support more than one listed species.

In recent years, freshwater inputs to the Salton Sea have declined sharply, with subsequent loss or modification of habitats. Fine salt and mineral particulates laden with environmental pollutants result in dust clouds with regional impact; the behavior of the dust clouds may be exemplary of movement of algal cells while at the same time the dust may limit movement of any released algae by covering/sedimentation. Area management projects designed to protect and improve Salton Sea habitats may play a role in algal dispersion and exposure.

*Geology*

The Salton Trough is a broad northwest-trending basin that represents the northward extension of the Gulf of California. The Imperial Valley is located in the southeastern half of the Salton Trough and is bounded by uplifted lacustrine sediments along the San Andreas Fault zone to the north, alluvial fans of the Chocolate Mountains to the east, dunes of the Sand Hills to the south, and the Salton Sea to the west. Tectonic activity that formed the trough continues at a high rate, as evidenced by deformed young sedimentary deposits and high levels of seismicity.[hh]

The Salton Trough is a topographic and geologic structural depression resulting from large-scale regional faulting. The trough is bounded on the northeast by the San Andreas Fault and Chocolate Mountains and on the southwest by the Peninsular Range and faults of the San Jacinto fault zone. **Figure H1** depicts the sites location relative to regional faults and physiographic features.

A geotechnical report was commissioned in 2011 by Energy Source LLC to evaluate the proposed Hudson Ranch II geothermal power plant on land west of English Road and South of McDonald road.[86] Much of this report's seismology findings are directly applicable to the land on which the SGI facility is sited due to its proximity. The primary seismic hazard is the potential for strong groundshaking during earthquakes along the Imperial, Brawley, and San Andreas Faults and the Brawley Seismic Zone. The project site does not lie within a State of California Alquist-Priolo Earthquake Fault Zone. Surface fault rupture is unlikely at the project site because of the well-delineated fault lines through the Imperial Valley as shown on USGS and CGS maps. The hazard of landsliding is unlikely due to the regional planar topography. No ancient landslides are

[gg] http://ca.audubon.org/mountain-plover-survey

[hh] https://maps.conservation.ca.gov/cgs/fam/

shown on geologic maps of the region and no indications of landslides were observed during the site investigations.

The EnergySource environmental report identified multiple seismic hazards. However, given that the algae cultivation ponds and evaporation pond utilize flexible polymer lining materials, secondary containment for ponds used for engineered algae cultivation, and that ponds are sited at- and slightly below-grade, and that the evaporation pond design meets current State design standards, the likelihood of a catastrophic failure which results in bulk cultivation or evaporation pond release is very low.

The USDA's online Soil Survey[ii] was consulted to determine the facility's soil composition. **Figure H2** provides the soil descriptions and boundaries. The site is comprised of two soil types: 114-Imperial silty clay, wet and 115-Imperial-Glenbar silty clay loams, wet, with a slope of zero to two percent. The evaporation pond is located within the 115-Imperial-Glenbar silty clay loams area. For a detailed analysis of the soil within the evaporation pond boundaries, refer to the geotechnical evaluation performed by TerraPacific Consultants where soil borings are reported.[87] Much of the ground surface of the western portion of the Salton Trough in Imperial County is covered by a veneer of recent sediments varying in thickness from zero to twenty feet. These sediments include eolian sand, as found in active sand dunes, and alluvial sand and gravel.

*Geotechnical study – soil permeability and groundwater*

A geotechnical analysis was commissioned to examine soil permeability.[87] Boring samples were taken to determine subsurface profiles at several locations within the north and south evaporation ponds. Borings exposed native lake bed deposit for the entire excavation depths. The lake deposits encountered are brown silty clay that was moist to very moist and soft to medium stiff in consistency. Very thin sand lenses (1/16-inch to 1/8-inch thickness) were also encountered within some of the borings. The soil profile which underlies the three evaporation ponds is comprised of cohesive low permeability sandy silt to sandy clay.

Permeability (hydraulic conductivity) testing was also conducted on *in-situ* samples. Samples included native lakebed deposits and remolded samples that were compacted to ninety percent of maximum density. Permeability rates (k) were measured to be all less than $1 \times 10^{-6}$ cm/sec. Soils with permeability measurements less than $1 \times 10^{-6}$ cm/sec are classified as low permeability.

A static groundwater table was not encountered within the 11.5-foot-deep borings; however, higher groundwater conditions can be developed at different levels within the soil profile due to variable bedding, future irrigation pattern, periods of prolong rain fall, seasonal fluctuations, and/or other conditions related to on or off-site development.

[ii] https://websoilsurvey.sc.egov.usda.gov/App/HomePage.htm

*Hydrology*

The facility site is located within the Salton Sea Transboundary Watershed (USGS Hydrologic Unit Code 18100200) and the Brawley hydrologic area of the Imperial hydrologic unit in the Imperial Valley Planning Area.

Surface water quality is a significant issue within and around the Salton Sea watershed. This watershed has been identified as a Category I impaired watershed under the 1997 California United Watershed Assessment.[jj] Poor surface water quality in the area is generally attributable to agricultural drainage containing high concentrations of nutrients and salts and to the discharge of the highly polluted New and Alamo rivers into the Salton Sea.

The area also receives most of its irrigation and potable water from the Colorado River through a series of canals diverted from the main branch of the Colorado River. The water quality of the Colorado River is degraded from its headwaters to its mouth by high salinity, carrying an annual average salt load of approximately nine million tons past the Hoover Dam, the uppermost location at which numeric criteria have been established.[kk]

The Salton Sea is a significant surface water feature that is located approximately 3.5 miles west and downstream of the facility site. It is one of the world's largest inland seas and is also one of the earth's lowest locations, at 227 feet below sea level (and rapidly falling). By virtue of its location in the Colorado Desert ecosystem, the Salton Sea receives minimal inflow from rain (average annual precipitation of 5.5 inches per year). The Salton Sea is mainly an agricultural drainage reservoir, a closed system with no outlet; ninety percent of the entire inflow is commercial agricultural runoff containing high concentrations of phosphates, nitrates, and salts from the Imperial, Coachella, and Mexicali valleys. Evaporation has caused the Salton Sea's salinity to increase and, as a result, water quality conditions continue to decline, and the Salton Sea cannot meet the beneficial uses assigned to it. The Salton Sea National Wildlife Refuge was designated in 1930, but recent bird die-offs suggest that declining water quality is adversely impacting avian populations.[ll] **Figure H3** identifies the streams and wetlands located near the project facility.

Basin management for the Imperial Valley is administered by the Imperial Irrigation District (IID). With more than 3000 miles of canals and drains, the IID is the largest irrigation district in the Nation. The IID water service area extends from the southern half of the Salton Sea to the U.S. – Mexico border. The IID Water Department is responsible for the timely operation and maintenance of the extensive open channel system and delivers up to 3.1 million AFY of IID's Colorado River entitlement to nearly 500,000 acres of irrigated land. Of the water IID transports, approximately ninety-seven

[jj] https://www.waterboards.ca.gov/coloradoriver/water_issues/programs/wmi/docs/saltonsea_watershed_staff_report.pdf

[kk] http://www.coloradoriversalinity.org/docs/2011%20REVIEW-October.pdf

[ll] http://www.parks.ca.gov/?page_id=639

percent is used for agricultural purposes. Water delivery for the SGI facility is provided via the O-Lateral canal.

*Minor drainage study*

The Minor Drainage identified 100-year storm volume conditions originating from on-site drainage areas tributary to the "N" Drain in the City of Calipatria, County of Imperial, California. The study identified the drainage areas and analyzed the runoff volumes due to a 100-year, 24-hour storm (three inches total rain). The site is located within the Imperial Irrigation District and is surrounded by a system of open channels and drains, which, from the analyses of site topography, aerial photography, and the FEMA map, are the only drainage features within the development area. There are no overland flows through, or offsite areas draining to this site.

The evaporation pond basins are adequate to retain the runoff volume produced from three inches of rainfall in accordance with the Imperial County Public Works Department (ICPWD) Engineering Design Guidelines Manual, Section III Drainage Improvements.[mm]

In addition, the study mapped the site outside of all special flood hazard areas and is given a designation as Zone X as shown on FEMA Flood Insurance Rate Map (FIRM) Panel 06025C0725C. Zone X is described as: "Areas to be outside the 0.2% (500 year) annual chance floodplain."

Considerable additional detail relevant to the project's on- and off-site hydrology, including engineering suggestions that were incorporated into evaporation pond design and grading plans, can be found in the Minor Drainage Study. **Figure H4** is a topographic map of the active south forty acres of the CAFF. There are a number of natural basins identified which collect rainwater. Two relevant basins for this TERA are those which would receive water from a loss of containment from either the 0.1-acre pond secondary containment area or the research greenhouse. Color-coded arrows denote the direction of drainage into their respective basins. The capacities of the "Greenhouse Basin" and "1-Acre Pond Basin" are 1.23 AF and 3.70 AF, respectively, are an order of magnitude greater than the anticipated engineered microorganism pond volumes. The time for basin water seepage into the soil or "drawdown" ranges from approximately one to two days.

In the unlikely event that there is a simultaneous loss of secondary containment for both 0.1-acre ponds along with a 100-year rain event, all pond water would drain into the 3.7 AF basin. In an abundance of caution, when either of the 0.1-acre ponds are in operation with an engineered microorganism, a plug will be maintained in the eight-inch drain pipe opening leading to the IID sump. In this

[mm] https://www.co.imperial.ca.us/publicwork/Forms/CountyProcedureManualSeptember2008.pdf

worst-case scenario, overflow from the 3.7 AF basin would be prevented from flowing into the IID drain to the Salton Sea.

*Groundwater*

The facility site lies within the Colorado Desert Province. The principal aquifer media in the Colorado Desert province are volcanic rocks, carbonates, and basin-fill sediments. Together, these aquifers are called the Basin and Range aquifer system. The Basin and Range physiographic province is classified at the regional level into hydrographic basins, depending on geologic drainage features such as the drainage boundaries of a large river or stream. Groundwater in the East Salton Sea groundwater basin moves from the recharge areas east of Imperial Valley and the Salton Sea toward the axis of the valley and converges upon the Salton Sea or the New and Alamo rivers. Recharge to the East Salton Sea groundwater basin is highly seasonal and comes primarily from runoff from surrounding mountain ranges.

There are no known groundwater wells within the facility site or the immediate vicinity.

Data on groundwater in the facility area are limited because there are few wells: groundwater in this part of the Colorado Desert and in the Imperial Valley is generally of poor quality due to high total dissolved solids (TDS) resulting from agricultural runoff, and well yields of clean water are quite low. Historically, there has been little need to investigate and develop the groundwater in the area due to the availability and low cost of imported surface water. Most studies of groundwater conditions in the central area of Imperial Valley focus exclusively on the upper 1,000 feet of water-bearing strata. Studies show that groundwater in the central part of the Imperial Valley generally occurs in two water-bearing zones: (1) a shallow (zero to 300 feet), unconfined aquifer that is bounded at depth by a low permeability clay (aquitard); and (2) an intermediate (300 to 1,500 feet), semi-confined aquifer that is bounded above by the aquitard and at depth by older marine and non-marine sediments. A third, deeper aquifer has been identified by some authors and may be present at depths of more than 1,500 feet, but it is likely not productive in terms of water supply resources. Typically, groundwater in the vicinity of the SGI facility is encountered at a depth of eight to ten feet below ground surface.

As stated earlier in the Soil Geotechnical Study section, a static groundwater table was not encountered within the 11.5-foot-deep borings.

*Existing nearby groundwater wells*

According to Imperial County Planning Development Services, there are no Conditional Use Permits (CUPs) or permitted drinking wells within a one-mile radius of the SGI facility. This was confirmed with the Imperial County Public Health Department. Subterranian water quality in the area quality is very poor and unsuitable for human consumption. Water sources for human consumption

are sourced from IID irrigation canals for subsequent treatment or by potable water delivered by truck. The nearby deep wells used in geothermal energy and mineral extraction are not suitable for potable water applications.

Local drinking water wells are highly unlikely to be contaminated from algae pond operations due to the absense of nearby wells, the low permeability of the soil beneath the evaporation pond liners and the impermeable nature of the synthetic pond liners.

*Facility water supply*

SGI has an agreement with IID to provide water. Annual water requirements are estimated to range from 100 to 500 AFY. This amount of water, available to the site via its agricultural water card, is likely sufficient for current and future R&D.

Water quality delivered to the site is monitored, at multiple canal system locations, by the IID on a yearly basis for California Title 22 compliance.

# I. Exposures of the Engineered Alga

A highly-detailed checklist for SGI's implementation of the NIH rDNA Guidelines for CAAF ponds subject to this application and for the CAAF greenhouse and photobioreactors is provided **(Table I1)**. Criteria listed in this table are addressed throughout this document.

A rubric for describing the practical implementation of biosafety and risk management from bioprospecting to commercialization is described in (**Figure I1)**: **"***Regulatory Learning Curve*". For the transition from wild-type to engineered microorganisms, it notes that physical containment such as biosafety cabinets, closed reactors, filters, ponds as well as handling practices are a function of accumulated biological, safety, health and environmental knowledge. EPA's regulations regarding engineered microorganisms reflects this process as implemented through the Toxic Substances Control Act (TSCA)[nn] and the Lautenberg Chemical Safety Act.[oo]

As strains undergo extensive laboratory and then outdoor environmental testing, and where their environmental safety profile continues to indicate low risk, they will advance through four different levels of containment:

1) Samples of unknown environmental composition are handled in the research laboratories under stringent NIH Guidelines Biosafety Level 2 (BL-2) conditions (USDA-permitted, dedicated negative pressure room, HEPA filtration, biosafety cabinet, with special personal protective equipment [PPE], decontamination, handling and waste disposal procedures) since they may contain insects, or human, animal, or environmental pathogens / toxins.
2) After strain isolation and identification, pure cell cultures are handled in the open research laboratories under NIH Guidelines Biosafety Level 1 (BL-1) for microorganisms unlikely to be pathogenic or toxigenic. The handling procedures are less restrictive and with simpler PPE requirements. For context, this is the level where most college freshman biology and microbiology laboratories are operated. Professional laboratories, such as the SGI labs in La Jolla, generally operate at a level approaching BL-2.
3) Greenhouses are operated under NIH Guidelines Biosafety Level 1 Large Scale (BL1-LS). We maintain open ponds in the greenhouses under the TSCA Biotechnology "inside a structure" R&D exemption and self-certify these activities, under the supervision of SGI's "Technically Qualified Individual (TQI)".[pp] Closed photobioreactors are also considered to be "within a structure". SGI greenhouses and PBRs are purpose-built to manage engineered microorganisms under BL-1 and BL1-LS conditions. They have special handling and containment measures designed to reduce the likelihood of microorganism release.
4) Outside of a "structure", such as in an open pond at the CAAF, SGI will manage engineered algal strains under the NIH Guidelines Good Large-Scale Practices (GLSP) criteria. In an abundance of caution, SGI will employ bird netting over both 0.1-acre ponds for this TERA. Also, there are detailed procedural controls, including SOPs for pond entry, sampling, cleaning, deactivation,

[nn] https://www.epa.gov/chemicals-under-tsca

[oo] https://www.epa.gov/assessing-and-managing-chemicals-under-tsca/frank-r-lautenberg-chemical-safety-21st-century-act

[pp] 40 C.F.R § 725.234 and 725.235. Activities conducted inside a structure.

liquid and solid waste disposal, and emergency response. SGI's TQI supervises all engineered microorganism handling activities at the CAAF site, including laboratory, greenhouse and ponds.

## 1) Occupational Exposure

The potential for exposure of the microorganism to workers has been qualitatively assessed for the workers in the facility as well as laboratory workers. Initial characterization of the workplace was done in the areas where microalgae are cultivated outdoors and the associated activities in the indoor laboratory. **Table I2** summarizes the observed tasks; protective equipment used, and task duration.

Exposure assessments are performed on groups of workers who carry out the same work activities in the same or similar work areas, and thereby have similar exposure potential to the hazardous agents found in those areas. The evaluation of the exposure potential via quantitative exposure monitoring is generally preferred but in the absence of a reference value (i.e., exposure limit), exposure index, or documented personal sampling strategy available, a qualitative exposure assessment is performed. A qualitative assessment reviews the activities performed by workers assigned to each group, collects information from several interviews with workers and supervisors, and observes the tasks. A detailed description of the framework used for this assessment is presented in the following sections. This assessment also allows for the identification of the factors that drive the exposure, which are the basis for the selection of any engineering or administrative controls.

In addition to the personal protective equipment used prevent contamination in the ponds and minimize dermal contact with the algae, there are various controls that minimize the exposure of workers and the environment to the algae, detailed in the following sections.

### I. Administrative controls

Administration controls such as the implementation of good work practices are standard throughout the facility. Workers are trained in the SOPs developed for each task in order to increase efficiency, minimize contamination of the ponds, prevent loss of product, minimize aerosolization of live or dead organisms, and prevent contact of personnel with microalgae or chemical substances (see **Appendices G3-G7**).

### II. Engineering controls

Engineering controls are also employed as part of standard work practices. These include:

1) design and implementation of automated systems wherever it is possible (such as measurement probes, remote sensing, and direct reading instruments) to minimize worker involvement in the process;

*2)* construction of the ponds within a secondary containment and a dedicated emergency chlorination system (see **Section G** for facility design and process description);

3) use of PBRs (enclosed process within the secondary containment) to grow algal cultures; and

4) use of laminar flow fume hoods in the laboratory to minimize possible exposures to algae liquid aerosols.

Given the implementation of engineering controls, administrative controls, and use of personal protective equipment, SGI believes that the occupational exposure to microalgae is minimal and mostly limited to the dispersion of microalgae in the air.

Similarly, such controls limit the release of algae to the environment due to the tasks associated with the cultivation of microalgae and the laboratory activities associated with such process.

## III. Qualitative Exposure Assessment

There are no OSHA (Occupational Health and Safety Administration) regulations that specifically address exposure limits for non-hazardous biological organisms, algae, or engineered microalgae. The specific agent that could be used in a meaningful way for a benchmark value is unclear (e.g., live algae, spores, etc.). However, given the nature of the work activities, the potential for exposure can be qualitatively assessed.

The qualitative rating for the exposure potential is developed using a "comparative model" where the observed exposure situation is compared to other operations the Industrial Hygienist has observed and for which measurable or observable exposure / environmental data are available. A detailed description of the qualitative assessment of the exposure potential is in Step 3 of **Appendix I1**. The approach takes into consideration the factors that:

1) generate the agents (e.g. physical / chemical / biological properties, process characteristics, etc.) in a form to which workers can be exposed (mists, particulates),
2) transport the agent to the worker (dilution in air, distance and direction to the receptor),
3) affect worker intake or contact with the agent (worker time and activity in the contamination zone, and controls), and
4) inform the industrial hygienist's estimate of potential exposure as compared to an established exposure limit (e.g. ACGIH TLV, OSHA PEL, etc.), if one exists.

When an established exposure limit does not exist, a surrogate exposure limit may be utilized where read-across to a meaningful limit is justified. Where no established limit or surrogate is available, the exposure component of the assessment may only be a relative ranking of activities with exposures as compared to one another. Since there is no exposure limit for exposure to algae, a relative scale of potential for exposure was assigned to each task. Section 3 of **Appendix I1** describes in detail the process and the factors that could affect generation, transport and contact with the agent. The resulting exposure potential can range from -6 (none) to 6 (the highest potential for exposure). **Table I3** lists the tasks and qualitative rating of potential for exposure for each of the tasks carried out at CAAF.

It is important to note that exposure potential only refers to the potential contact with the organism rather than any hazard or risk. This qualitative assessment of exposure potential is a conservative approach to identify those tasks where workers could come into contact with the microalgae. In **Table I3,** the tasks with greater potential for exposure (sample collection; pond monitoring and experimental termination) have rankings of 3, and 2 respectively, which indicates that although the potential for contact with algae through inhalation exists, it is expected to be low to moderate.

Although the best professional judgment has been applied to the evaluation of the potential for exposures, the observers have limited understanding of the processes that generate and transport the agent, as the characteristics of the agent (such as dispersion and transport) are not fully understood. To account for these uncertainties, the assessment followed a very conservative approach based upon relative potential for exposure to algae, considering worst-case scenarios. Considering that there is no known previous exposure assessment for algae operations, no reference value for algae, and no exposure indicator for algae, the qualitative exposure assessment presented in **Table I3** conservatively concludes that the majority of the tasks have relatively low exposure potential, and only a few short-duration tasks have relatively moderate potential for exposure.

This qualitative exposure assessment, along with the hazard characterization of the algae is used to carry out a qualitative risk assessment (see **Table I4**). This assessment results in a matrix that combines the potential for exposure to any particular material and the material's potential effects on human or environmental health (hazard category ranking). **Table I4** presents the moderate potential for tasks assessed at the CAAF facility rated in terms of the qualitative ranking of exposure potential.

The conservative qualitative exposure assessment leads to the conclusion that the potential for exposure in a few activities associated with a selected task is moderate at the most (i.e. a score of 3) and the hazard category based in the human health effects is low (category IV). Therefore, in the worst-case scenario of moderate potential for exposure, the potential for a health effect (the health risk) is negligible when operating at normal conditions.

## 2) Environmental and General Population Exposures

### I. Environmental releases from commercial facilities to various media

As discussed above and in **Section G**, there are numerous design and operational practices at the CAAF that provide containment and prevent offsite dispersal of microalgae. These include secondary containment, pond liners, chlorination facilities, and high-level pond management to prevent overflows. These design and operation practices primarily target movement of microalgae via water; however, aerosol formation is also a potential source of microalgae dispersion CAAF. Though overall minimal, the formation of aerosols is most likely to occur from the paddlewheels as they move in and out of the water column and through aeration/carbonation of the ponds. Aerosol formation can also occur due to splashing during movement and transfers of microalgae laden water. Pond movements (i.e. transfers and filling) are conducted through pipes/hoses, to eliminate splashing into the pond. To minimize aerosolization and spills (and therefore potential for exposure), engineering controls and practices are implemented, such as:

1) low pressure operation such that splashing is minimized;
2) no high-pressure hoses are used for transfers;
3) all microalgae and lab materials exposed to microalgae are treated with bleach or autoclaved prior to disposal or reuse;
4) human exposure is minimized by assuring cells are dead prior to cleaning the ponds,

5) introduction of gases into the ponds is done by means of micro-aeration, so as not to cause large bubbles, and as such, minimize aerosol formation; and

6) good microbiological practices for transfer and sampling have been adopted as best practices.

Our ecological risk assessment (ERA) incorporates a characterization of the fate or persistence of the subject GE alga (presented in greater detail above in **Section F**) along with a discussion of potential transport mechanisms. A conceptual site model for transport and potential ecological receptors is presented in **Figure I2**, but by no means are the modeled transfers or exposures believed to be significant.

II. Transport

Microalgae transport from CAAF to environmental media and receptors is anticipated to occur even with engineering controls implemented to minimize release. The use of open ponds ensures contact between the culture medium and the ambient air, which may result in the formation of aerosols which are then transported from the pond area to environmental media. While bird-netting will be used for the proposed experiments, it may not exclude all wildlife (e.g. flies and other insects) which could contact and subsequently carry algae from the site in or on their bodies. Algae from operations or aerosols may settle on the soil or on dusty facility surfaces and then become airborne if disturbed.

There is an abundance of published studies seeking to quantify exposure to hazardous algal blooms; however, most of these measure algal toxins, (e.g. brevitoxins), rather than aerosolized algae because the toxin is the substance of concern. These studies are thus of little use in estimating transfer of algae to the air via aerosolization. It has been postulated that the size and life span of atmospheric aerosols resembling inverted micelles is similar to that of microbes (bacteria).[88] It is expected this pathway will represent minimal exposure due to the limited travel distance of aerosol droplets relative to the expected distance of most receptors from the site, and the size of the algal cells limiting the inhalation of the cells into the alveolar space. Birds coming in close contact to the facility ponds could experience inhalation of the engineered algae in aerosols, similar to their exposure to natural species in other aquatic environments.

Several studies have evaluated transport of microalgae by aquatic dipterans (craneflies, mosquitoes, midges and horseflies), and birds, respectively, and discovered numerous viable green algae and cyanobacteria were carried on, or in, their bodies with the potential to colonize water bodies some distance from their point of collection based on the estimated travel distance of aquatic insects and migratory birds.[89-91] Another study evaluated viable species of algae and protozoa in the atmosphere downwind of Lake Michigan, by bubbling ambient air through sterile pond water for varying amounts of time (up to 32 hr.)[92] It was found can be generally good survival under varying humidity and wind conditions (up to fifteen mph, the highest tested). Most of the organisms found were *Chlorella spp*., several of which species are recognized as colonizers of drier, terrestrial surfaces. As described for other aquatic green algae[93] and shown in our own experimental data, we have learned that the recipient and subject strain are highly susceptible to desiccation (**Figure F5**).

III. Exposure routes and summary of receptors impacted.

*Air/Inhalation*

Terrestrial receptors may be exposed to airborne (particulate or aerosol) algae; it is expected this pathway will represent a minor pathway for exposure due to the limited travel distance of aerosol droplets of airborne algae. The amount of aerosol is expected to be low based on the site physical and operational conditions. Birds coming in close proximity to the facility ponds could experience inhalation of the engineered algae in aerosols, similar to their exposure to natural species in other aquatic environments. Air-breathing organisms living at the air-water interface (e.g., water fowl, amphibians) may experience greater exposure to aerosolized cells, but such interactions are expected to be very low due. Aquatic organisms are not exposed to air per se but to dissolved gases in water. Dust inhalation may be another exposure route; however, based on the environmental fate information above, any aquatic microalgae which become desiccated and thus incorporated into dust are unlikely to remain viable under many atmospheric conditions.

*Direct contact and ingestion*

Terrestrial receptors may be exposed to algae on soil surfaces, and wind-borne dust (deposited from air); this exposure rate is expected to be low and the viability of the algae on these surfaces may be limited by drying and by soil characteristics (see ecological fate discussion and test results above). Terrestrial soil-dwelling organisms may receive greater direct contact and ingestion exposure. In aquatic environments, introduced algae may proliferate in the water column as well as on sediment and rock surfaces resulting in ambient exposure of aquatic life such as rooted aquatic vegetation and benthic (infaunal or epifaunal) invertebrates, and bottom-feeding fish. Likewise, fish, benthos, and aquatic plants are directly in contact with surface water. Riparian birds and mammals (those active at the water's edge), and insects, may have direct contact with surface water (including flying insects and birds using the aquatic habitat of CAAF). Introduced algae which proliferate in irrigation waters may end up in terrestrial environments. However, this route is expected to be a minor pathway.

In summary, the risk to ecological receptors including domestic animals, crop plants and wildlife during experimental cultivation is low based on facility containment, limited exposure pathways and anticipated exposures, and the absence of toxins or infections produced by the selected algal strains; however, the potential for exposure to aquatic and riparian organisms exists (**Table I5**).

IV. Inactivation methods and pollution control technologies

Inactivation studies were performed in order to determine effective inactivation methods. The full SOP is attached in supplemental material (**Appendix I2**). Briefly, cultures were inoculated at an OD of approximately 1.0 and were exposed to varying concentrations of toxicant for one, two, or four hours. At the end of the toxicant contact time, the vessels were centrifuged to remove any extracellular toxicant, and the pelleted biomass was utilized to inoculate cultures into fresh media. These cultures were incubated for one week before examining for growth. An

inactivation method was deemed to be effective if after one week of growth, no viable cells were observed in the new culture vessels.

Inactivation studies were performed on STR00010, STR00012, and STR26155. Experimental data showed that 2 mL/L of 4.0% sodium hypochlorite was sufficient to inactivate STR00010 after one hour. STR00012 and STR26155 were inactivated with 1 mL/L of 4.0% sodium hypochlorite after one hour with good mixing (**Table I6**). All SGI protocols for inactivation utilize at least 4 mL/L of 12.5% sodium hypochlorite and a minimum contact time of 1 hour to ensure a total deactivated before disposal. Thus, standard SGI CAAF protocols apply greater than a 12.5-fold excess hypochlorite treatment (than that experimentally determined) to inactivate the subject strain providing a conservative treatment for algal cultures.

Clean-in-place procedures are utilized for cleaning ponds at the CAAF site. At the conclusion of an experiment, ponds are scrubbed along the sides with brushes to remove any films that may have formed over the course of an experiment. Then, ponds are dosed with 4 mL/L of 12.5% sodium hypochlorite and thoroughly mixed with the in-pond paddlewheels. After at least one hour, and after complete mixing, the ponds are then pumped directly to the on-site evaporative disposal pond via a dedicated line.

## V. Environmental exposures

*Proximity to surface water bodies*

The facility site is located within the Salton Sea Transboundary Watershed (U.S. Geological Survey [USGS] Hydrologic Unit Code [HUC] 18100200) and the Brawley hydrologic area of the Imperial hydrologic unit in the Imperial Valley Planning Area.

Surface water quality is a significant issue within and around the Salton Sea watershed. This watershed has been identified as a Category I impaired watershed under the 1997 California United Watershed Assessment. Poor surface water quality in the area is generally attributable to agricultural drainage containing high concentrations of nutrients and salts and to the discharge of the highly polluted New and Alamo rivers into the Salton Sea.

The area also receives the majority of its irrigation and potable water from the Colorado River through a series of canals diverted from the main branch of the Colorado River. The water quality of the Colorado River is degraded from its headwaters to its mouth by high salinity, carrying an annual average salt load of approximately 9 million tons past Hoover Dam, the uppermost location at which numeric criteria have been established.

The Salton Sea is a significant surface water feature that is located approximately three miles west and downstream of the facility site. It is one of the world's largest inland seas and is also one of the earth’s lowest locations, at 227 ft below sea level. By virtue of its location in the Colorado Desert ecosystem, the Salton Sea receives minimal inflow from rain (average annual precipitation of 5.5 inches per year). The Salton Sea is mainly an agricultural drainage reservoir, a closed system with no outlet; ninety percent of the entire inflow is commercial agricultural runoff containing high concentrations of phosphates, nitrates, and salts from the Imperial, Coachella, and Mexicali valleys. Evaporation has caused the Salton Sea’s salinity to increase and, as a result, water

quality conditions continue to decline, and the Salton Sea cannot meet the beneficial uses assigned to it. The Salton Sea National Wildlife Refuge was designated in 1930, but recent bird die-offs suggest that declining water quality is adversely impacting avian populations Salton Sea state recreation.[qq] **Figure H2** identifies the streams and wetlands located near the project facility.

*Proximity to migratory bird routes*

The County of Imperial is located on the Pacific Flyway for migratory waterfowl, shorebirds, and songbirds. Although this area is considered to be part of the Colorado Desert, approximately 500,000 acres of the Colorado Desert in the County of Imperial, including the facility site, have been converted to agricultural use. The irrigation system in the Imperial Valley attracts many bird species that are typically found in agricultural areas, including waterfowl, gulls, herons, cranes, ibises, egrets, doves, quail, sparrows, juncos, and finches. Some raptor species forage in this area as well, particularly the Western Burrowing Owl (*Athene cunicularia hypugea*), which also uses burrows in many of the irrigation canals and drains.

Further details for the CAAF site and surrounding areas are presented above in **Section H4**.

Receptors of potential concern, or ROPC, are representatives of various trophic or taxonomic categories which may come into contact with an environmental stressor. Using the conceptual site model (**Figure I1**) to depict routes of exposure, representative receptors likely to be exposed have been identified for risk assessment.

Within the CAAF vicinity, aquatic environments are those most likely to support growth of microalgae. Categories of potential receptors include benthic/water column crustaceans (barnacles and amphipods); wading, diving, and waterfowl; and fish in the highly saline Salton Sea. Similar types of birds and fish, in addition to amphibians and reptiles (turtles), as well as aquatic insects, would be expected to occur in the fresh/brackish water resources of the constructed water bodies (irrigation laterals and drains, constructed wetlands, and open-pond cultivation systems of the near-field CAAF environment). Riparian mammals such as raccoons also may use the aquatic habitats, and terrestrial insects including pollinators are abundant in the cultivated and desert habitats.

## VI. General population exposures

*Inhalation Exposure*

As described in **Section H**, the site is located in an agricultural and industrial area, at a distance of at least 3.5 miles from any population centers. Inhalation exposure is not assumed to be limited to the work site; small quantities of microalgae may become airborne or be carried off-site by wind or wildlife. These exposures are expected to be

[qq] http://www.parks.ca.gov/?page_id=639

*de minimis*; exposures to these concentrations are applied equally to the general population and to ecological receptors as both are anticipated to be equally exposed.

*Drinking Water Exposure*

This exposure pathway is not considered to be relevant.

The drinking water system for Imperial Valley depends solely on the Colorado River, which is dammed at the Imperial Dam, located more than 50 miles east of the site. The city of Brawley is the closest water treatment plant and is located 15 miles south. Given the wind direction prevalence (west and west-southwest), the potential exists for a bioaerosol to be transported outside of the facility. However, given the distance of the treatment plant and the dam, likelihood of this bioaerosol reaching these sites is minimal. Furthermore, the water treatment and chlorination are likely to inactivate any microalgae before it can be distributed for ingestion.

*Proximity to the general human population, urban centers, schools, etc.*

As described in **Section H**, the closest population centers are the towns of Brawley (located fifteen miles south of the site, approximate population: 26,500), Calipatria (located six miles southeast of the site, approximate population: 7,500), and Niland (located 3.5 miles northeast of the site, approximate population: 1,000). Given this distance and the prevalent wind direction, exposure to general population is expected to be *de minimis*.

*Proximity to aquaculture farms, agricultural crops/poultry/livestock*

As described in **Section H**, there are active hunting and fishing clubs immediately south and southwest of the facility, the site is in close proximity of agricultural lands, and within twenty miles of livestock farms. There is one commercial aquaculture operation located nearby (Earthrise Nutritional, ~3.5 miles from CAAF). Last, there are some areas of water recreational use, such as the Salton Sea and the Sonny Bono Salton Sea National Wildlife Refuge. All of these areas could be considered natural receptors of the aerosolized algae, although, given the distance from the site, exposure is expected to be *de minimis.*

*Assessment of exposure to the general population*

There are no regulations that specifically address general population exposure as related to microalgae organisms; there are no known exposure limits to algae or engineered microorganisms. Therefore, a qualitative exposure assessment that uses the same methodology as the qualitative exposure assessment for the worker/occupational population will be used; evaluation of the same factors that affect generation, transport, and contact with algae will be examined. This qualitative assessment is conservative in nature and assumes that the point of exposure is immediately downwind of the facility and at the fence line.

**Table I7** summarizes the qualitative exposure assessment completed for the general population and environment and includes the associated intensity modifier category. The last column summarizes the qualitative assessment for the potential exposure for

the general population or the environment. Additionally, the table presents an unlikely case in which there is an uncontrolled release of the organisms due to a severe weather event with no warning. However, such an event has not occurred during the seven years SGI has owned the facility.

For the general population risk assessment (**Appendix I1**), the two cases were considered: potential exposure to the general population through normal operations (qualitative exposure potential = 1) and potential exposures to the general population due to an unforeseen weather event (qualitative exposure potential = 3). Note that similar to the worker risk assessment, the hazard category based in the human health effects is low (IV).

### 3) Consumer Exposures

The objective of this activity is to develop the technology to produce biofuels. There is no expected consumer use for this TERA strain or for future TERA strains. The algae will be deactivated on-site.

# J. Monitoring of the Engineered Alga

## 1) Monitoring Endpoints and Procedures

### I. Endpoints that will be evaluated in samples that are collected

It is acknowledged that algae can disperse through the aerosolization of the culture medium in an open raceway pond due to typical processes such as paddle-wheel movement, air-$CO_2$ injection and bubbling, and general splashing at the air-water interface. We have obtained preliminary data where we have observed that SGI production algae are detectable at very low levels in bio-aerosols collected near our open raceway ponds. During the proposed TERA experiments, periodic bio-aerosol sampling will be conducted, and samples assayed for quantification of the subject alga. These data might provide insight into the total emissions from a pond. Collecting these data concurrent with the samples described below also might provide insight to both total emissions from the ponds, as well as the characterize the ability of the emitted algae to disperse and establish at a distance.

One primary means to assess the potential of the subject alga to not just disperse, but also establish itself will be through the establishment and regular sampling of "algae traps" around our experimental ponds. The algae traps are made from commercially available 100-gallon stock tanks. Circulation is maintained through the use of solar powered pumps. The trap ponds will be filled with the standard artificial seawater media used at the CAAF site and topped off weekly with freshwater to account for evaporative losses. Four primary trap ponds will be positioned at intercardinal directions at a distance of ~150 m from the experimental ponds (**Figure J1**). A fifth trap pond will be established downwind of the prevailing winds at the furthest distance possible on our property. This is approximately 550 m from the experimental ponds.

In addition to these purpose-built trap ponds, we will also use all additional active ponds on site as potential receivers of the subject strain to be sampled and assayed for the subject strain. These include one-acre ponds immediately North of the experimental ponds ranging from 20 m to 100 m away. There are an additional twenty raceway ponds to the east-southeast of the experimental ponds which range in size from 0.1 acre down to 2 $m^2$. These ponds range from 200 m to 250 m from the experimental ponds. While operational plans will dictate which ponds are in active use during the TERA experiment, there will undoubtedly be at least ten or more raceway ponds to sample from.

Lastly, both soil and water samples from our established sampling stations (see **Section F** for details) during the course of the experiment and for a full year from the start of the experiment. These samples provide a means to monitor the local environment for the potential dispersal and establishment of the subject alga outside of our facility.

### II. Techniques used to detect the microorganism in test samples

Molecular methods will be used to assay the collected samples for the presence and abundance of the subject microbe. In both cases we will be relying on diagnostic nucleic acid sequences for the identification of the subject *Parachlorella* STR26155.

One method we will employ is microbiome profiling. In our approach we amplify a highly conserved taxonomic marker, specifically a portion of the SSU rDNA gene, from metagenomic samples for subsequent sequencing and classification. We employ Illumina sequencing technology to generate tens of thousands of sequences from each sample. There are high-quality, well-characterized, and curated databases of SSU rDNA sequences that can be used to taxonomically classify these reads resulting in a comprehensive identification and semi-quantitative accounting of all microbes in a sample.

In addition to microbiome profiling we have developed a qPCR assay for the subject alga. This method relies on the absolutely unique DNA signature that is present only in our engineered alga amplifying a sequence which encompasses the 5' end of the introduced gene cassette and the flanking genomic region.

*Sensitivity and reliability of the method and the actual limit of detection*

The qPCR method we have developed is highly sensitive, providing a linear range of quantification that spans 5 orders of magnitude and able to reliably detect down to one genome equivalent (**Figure J2**). This provides a strong foundation for highly specific quantification of the subject strain; however, this is under ideal analytical conditions. To further assess the actual limit of detection for the subject engineered alga we conducted a spiking experiment. Using water collected from the Salton Sea, we spiked the subject alga into triplicate 100mL water samples at each of six levels (i.e. 0.1 cell/mL, 1 cell/mL, 10 cells/mL, 100 cells/mL, 1,000 cells/mL, 10,000 cells/mL, and a "no-spike" negative control). The qPCR method reliably detected the subject strain at 1 cell/mL and was clearly above any background detection at 10 cells/mL (**Figure J3**).

Microbiome profiling methods were also highly sensitive, albeit somewhat less so than the qPCR method. Sensitivity in these sequence-based approaches are directly dependent on sequencing depth. Our standard for these methods is to generate >25,000 reads per sample. At this sequencing depth our spiking experiment reliably detected the subject strain at 10 cells/mL and was clearly above any background detection at 100 cells/mL (**Figure J3**). This approach provides a wealth of data on each sample but does not detect the subject strain exclusively. As the marker sequence used in these analyses are unchanged in wildtype and all derived engineered strains, this method will be unable to distinguish recipient from subject strain. While this is true, we still believe that these analyses are highly informative, with the additional benefit of being relatively high-throughput. Moreover, we have been conducting monthly sampling for environmental monitoring at our established sampling stations for a full calendar year, initiated in February of 2018, starting with and then adding additional locations where SGI has obtained legal access.

Serendipitously, we have never detected the sequence variants from SGI's *Parachlorella* in the environmental samples. This indicates there are no wild-type strains present in the environment with identical SSU rDNA sequences to SGI's proprietary *Parachlorella*. While the detection of SGI's *Parachlorella*-specific SSU rDNA sequences in a microbiome dataset does not affirmatively detect the subject strain, it will allow us to quickly triage

which samples will be re-tested with the more sensitive and subject strain specific qPCR method.

*Frequency and type of observations to be made*

See **Table J1**.

## 2) Sampling Procedures

### I. How, where, and when samples will be taken for each monitoring endpoint

We will conduct active monitoring for one week prior to the start of open engineered alga cultivation, during the entire course of the experiment and for two weeks following termination of the engineered alga ponds. During this active monitoring period all sample types (bio-aerosols, trap ponds, CAAF ponds, and environmental sampling stations, see **Table J1**) will be collected. In addition, environmental samples from our established sampling stations will be conducted monthly for one year from the start of the experiment.

Bio-aerosol sampling will be conducted using Bobcat™ air samplers (Innovaprep, Drexel MO). On a weekly basis, these samplers will be positioned alongside the four primary algae traps and operated according to manufacturer's recommended protocols. Following the air collection, particles collected on the electret filters will be eluted using a tris-based buffer "rapid filter elution kit".

Water samples (from trap ponds, CAAF ponds, and sampling stations) are to be collected in sterile 500 mL bottles.

Soil samples are collected in 15 mL or 50 mL tubes.

*Standard procedures for preserving, processing, and analyzing samples*

1) All samples will be maintained on ice or in 4 °C refrigerators during collection and transport to the lab.
2) Eluted bio-aerosol samples are ~6 mL and typically rather dilute. In order to ensure minimal losses these samples are further concentrated using 50 MWCO (molecular weight cut-off) centrifugal filters (Millipore, Burlington MA). The resulting ~200 µl can be directly subjected to DNA extraction methods. DNA from these concentrated bio-aerosol samples as well as soil samples are extracted using the Qiagen DNeasy™ Powersoil™ kit (Hilden, Germany) according to manufacturer's protocols.
3) Biomass from water samples are further concentrated using 0.2µm vacuum filtration (Pall, Port Washington NY). Up to 500 mL is concentrated, depending on the sample type, as some high-biomass samples rapidly foul the filter surface. The volume of water sample filtered is recorded so that calculations can be made as to the concentration of organism per sample volume. DNA from biomass-containing filters are subjected to DNA extraction using Qiagen DNeasy Powerwater™ kit (Hilden, Germany), again according to manufacturer's protocols.

*Methods of measurement, equipment, precision bias, accuracy, repeatability and statistical analysis of the methods*

The primary means of measurement of the subject strain in the various sample and experiment types are molecular. One method we employ is quantitative PCR using primers which specifically target the genome-gene cassette junction created in the subject strain. This method employs the empirical and statistical methods outlined by Bustin *et al.*[94] For all qPCR measurements, we perform technical triplicate reactions. The standard curves for qPCR detection and quantification of the subject strain referenced above show sensitivity down to near single cell (in this case near single gene copy) with a correlation coefficient > 0.99 and a linear range spanning five orders of magnitude.

Another means of molecular detection of the subject strain amidst a complex microbiome employs amplicon sequencing. These methods generate tens of thousands of individual sequence reads from a taxonomically informative genetic sequence (the SSU rRNA gene for which high-quality curated databases are available). The field of microbiome sequencing/profiling is rapidly advancing. As such, the tools for sequence analysis and classification are also rapidly changing. We utilize a proprietary bioinformatic workflow which uses a sequence de-noising approach with the QIIME[rr] (quantitative insight into microbial ecology) open-source software package.

[rr] http://qiime.org/

# K. Termination and Emergency Containment Procedures

## 1) Type of unexpected effects that would necessitate the emergency termination of a field test or environmental use

As discussed in **Section G.9** above, our monitoring plan will be in-place to detect the possible dispersal, establishment, and proliferation of the subject alga.

### II. Detection during routing monitoring

If the subject strain is detected in any algae trap or wild-type or classically-improved production ponds on-site, follow-on sampling will continue.

If we detect a 1,000-fold increase of the subject microorganism (by qPCR) from its originally detected titer, this would indicate that the subject strain has established itself and is actively proliferating. In this instance, additional confirmatory testing would be performed. Upon confirmation, SGI management will contact EPA's Biotechnology Program office within five (5) business days for further consultation.

### III. Potential for loss of containment

In the event of unexpected weather, seismic event or other circumstance where a loss of secondary containment might result in a bulk release of engineered algal cultures to land or to local waters, decision-making authority is given to site management to immediately initiate emergency termination procedures.

In the highly-unlikely event of loss of secondary containment resulting in substantial discharge to land and/or local waters, SGI will immediately initiate emergency containment and inactivation procedures. Once emergency procedures are underway, then within one (1) day, SGI management will coordinate with EPA's Biotechnology Program office on the emergency termination efforts.

## 2) Emergency termination procedures to be followed if adverse environmental effects are observed

Four 55-gallon barrels of concentrated bleach (12.5% sodium hypochlorite), containing sufficient material to inactivate twice the production capacity of all the ponds and PBRs located within secondary containment area, will be stored immediately adjacent to the containment area. In the event of adverse environmental events or unanticipated emergencies, the experiment will be rapidly terminated by pumping the bleach into the appropriate pond or PBR per the applicable SOP.

## 3) Handling of spills or leaks

Spills from 0.1-acre ponds and PBRs will be quantitatively held within secondary containment, treated with bleach, and subsequently discharged into the evaporation pond per the applicable SOP.

## L. Record Keeping & Reporting of Test Results

SGI will maintain records for three years after completion of the project described in this TERA, in accordance with 40 C.F.R. § 725.250(f)(1). SGI will provide summaries of all data, conclusions, and reports within one year of the end of the project described in this TERA, in accordance with 40 C.F.R. § 725.250(f)(2).

# List of Tables

# List of Figures

| Figure |
|---|
| **Figure F 2: Aerial view of SGI's CAAF facility and local vicinity** |
| **Figure F 3: Growth of wild-type Parachlorella STR00010 and recipient strain STR00012 in sterile-filtered waters** |
| **Figure F 4: Growth of recipient strain STR00012 and subject strain in sterile-filtered waters** |
| **Figure F 5: Desiccation tolerance of recipient strain STR00012 and subject strain STR26155** |
| **Figure F 6: Whole-culture primary productivity measurements for Salton Sea, managed marsh waters, Morton Bay and duck/bass ponds spiked with recipient strain, subject strain with negative controls in unfiltered local waters, collected in May/June 2018** |
| **Figure F 7: Microbiome profiling data for invasion expt. using waters collected from Morton Bay and duck/bass ponds** |
| **Figure F 8: Whole-culture primary productivity measurements for Salton Sea and managed marsh waters, spiked with recipient strain, subject strain with negative controls in unfiltered local waters, collected in August 2018** |
| **Figure F 9: Whole-culture primary productivity measurements for Salton Sea and managed marsh waters spiked with subject strain at three inoculation densities with negative controls, collected October 2018** |
| **Figure F 10: Microbiome profiling data for ALBOUT_0040** |
| **Figure G 1: Satellite photo of CAAF facility (Feb-2015) designating key structures and facilities relevant to the application** |
| **Figure G 2: Summary of prevailing winds at CAAF site from the Brawley CIMIS (2012) and CAAF weather stations (2018)** |
| **Figure G 3: Process-waste evaporation-pond-liner engineering detail** |
| **Figure H 1: Geologic faults map of Salton Sea and areas surrounding CAAF facility and the Imperial Valley** |
| **Figure H 2: Soil categories, streams and wetlands on the CAAF site and adjacent areas** |
| **Figure H 3: Topographic map of project area with neighboring municipalities, roads, water and geothermal features** |
| **Figure H 4: CAAF drainage study denoting retention basins and drainage** |
| **Figure I 1: Regulatory Learning Curve for wild-type, classically-improved and engineered microorganisms.** |
| **Figure I 2: Conceptual transport model and potential exposure of ecological receptors to microalgae for the CAAF** |
| **Figure J 1: Identification of the facility environmental sampling locations** |
| **Figure J 2: qPCR standard curve specific for the subject alga quantification** |
| **Figure J 3: Detection and quantification of the subject alga in a complex environmental sample (Salton Sea water) by two orthogonal analytical approaches – qPCR and metabolic profiling** |

# List of Appendices

| Appendix | Corresponding Filename |
|---|---|
| Appendix A1: Nucleotide sequence of SGI Parachlorella sp. SSU rRNA | No-CBI Appendix A1 SGI Parachlorella SSU rRNA Sequence Data.txt |
| Appendix C1: Nucleotide sequence of NAS14335 | No-CBI Appendix C1 NAS14335 Sequence Data.txt |
| Appendix C2: Nucleotide sequence of RS1 insertion site region | No-CBI Appendix C2 RS1 Insertion Site Sequence Data.txt |
| Appendix D1: Results of AllergenOnline database queries | No-CBI Appendix D1 Results of AllergenOnline Database Queries Performed 22-Feb-2019.pdf |
| Appendix I1: Health and Environmental Assessment – Supporting Information | No-CBI Appendix I1 Health and Environmental Risk Assessment Supporting Information.pdf |
| Appendix I2: Strain Inactivation SOP | No-CBI Appendix I2 Strain Inactivation SOP.pdf |

# List of Supplemental Files

| Description | Corresponding Filename |
|---|---|
| This document | No-CBI SGI GFP-Parachlorella STR26155 TERA Main Document 07-Apr-2019.pdf |
| Tables and Figures of the main document | No-CBI SGI GFP-Parachlorella STR26155 TERA Figures and Tables 07-Apr-2019.pdf |
| Papers referenced in the main document | Mailed to EPA on CD. |
| *Chlorella* SH&E review[30] | Buxser, S. (2010) Chlorella Safety Health and Environmental (SHE) Literature Review and Hazard Assessment.pdf |
| Papers referenced in the *Chlorella* SH&E review[30] | Mailed to EPA on CD. |
| *Parachlorella* SH&E review[32] | Buxser, S. (2019) Parachlorella Safety Health and Environmental (SHE) Literature Review and Hazard Assessment.pdf |
| Papers referenced in the *Parachlorella* SH&E review[32] | Mailed to EPA on CD. |

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